Alan Javier Hernández-Álvarez
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Green Protein Processing Technologies from Plants

Novel Extraction and Purification Methods for Product Development

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Preface

Global demand for plant protein is growing due to multiple factors, including an expanding population and changing consumer trends. CO_2 emissions, water requirements, and nitrogen footprints of plant production are often lower than those of livestock farming, and these high sustainability gains combined with low technology requirements make plant proteins an attractive investment for producers and processors. Plant protein ingredients such as flour, protein concentrates (65–90% protein dry basis), protein isolates (90% + protein dry basis), partial and extensive protein hydrolysates, as well as their derived food products (bread, pasta, cookies, etc.), are widely available to consumers. The sources of plant protein are incredibly varied and include cereals, oilseeds, pseudocereals, pulses, and their processing by-products. At an industrial scale, plant proteins are extracted by alkaline extraction and are recovered by isoelectric precipitation. The main advantages of this process are its high productivity and its easy scalability. However, plant protein extraction can be limited by the interactions between the proteins and other components, such as lipids, polysaccharides, and polyphenols present in the plant cells. For that reason, cell disruption is the initial phase to allow protein release from the plant cells.

Traditionally, it has been performed by mechanical methods such as grinding and milling. However, novel processing technologies are emerging to improve plant cell disruption and, consequently, protein extraction. Another concern with conventional processes is the use of harsh chemicals for both the extraction and the isoelectric precipitation steps. The use of these chemicals has a negative impact on the functional and organoleptic properties of the resulting ingredients, as well as having a deleterious impact on the environment. Therefore, emerging purification technologies that have a lesser impact on protein functionality, and on the environment, have been investigated.

This book discusses a wide range of processing methods from the traditional air classification and alkaline extraction-isoelectric precipitation to more modern technologies such as reverse micelle extraction and ultrasound-assisted extraction. We believe that the discussion of these methods, as well as their effect on protein functionality and nutritive quality, over a series of 13 chapters will be of significant benefit to industry professionals, researchers, undergraduate and postgraduate students (including research students) in food science and technology,

biotechnology, nutrition and engineering, and many other individuals interested in optimizing the use of plant-based proteins.

We are grateful to the many authors who contributed chapters to this book, as well as the helpful staff at Springer for the support in bringing this book to publication.

Leeds, UK — Alan Javier Hernández-Álvarez
QC, Canada — Martin Mondor
Guelph, ON, Canada — Matthew G. Nosworthy

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- Prof Caroline Orfila, Ph.D.—School of Food Science and Nutrition, University of Leeds –Professor of Plant Biochemistry and Nutrition.
- Assoc Prof Christine Bosch, Ph.D.—School of Food Science and Nutrition, University of Leeds—Associate Professor in Nutrition.

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About the Editors

Alan Javier Hernández-Álvarez is a biochemical engineer by training, with a Ph. D. in Food Science and Nutrition (2012, National Polytechnic Institute, IPN) - Fat Institute, CSIC, Seville, Spain. He worked as a Lecturer at the Food Biochemistry Faculty, Biochemical Engineering Department at the National Polytechnic Institute, Mexico from 2012 to 2013. Awarded a postdoctoral fellowship from CONACyT in 2013 to follow his research at Agriculture and Agri-Food Canada, Saint-Hyacinthe, Quebec, Canada. After this, he was awarded a Visiting Fellowships in Canadian Government Laboratories Award, NSERC, Canada, 2014–2017. During his time at Agriculture and Agri-Food Canada, he worked with a wide variety of research projects in the areas of value-added food processing, and food quality, acting in an advisory and research capacity providing expertise on protein quality assessment, protein extraction and characterization, techno-functional properties assessment and bioactivity of proteins/peptides. He has collaborated with different Canadian/UK Industry Companies in a wide variety of industry-based projects such as Nature Zen, InnoVactiv, SEMICAN, THIS, Meatless Farm among others. Currently a Lecturer in Nutrition and Global Health at the School of Food Science and Nutrition, University of Leeds since 2019. Member of the Global Food and Environment Institute (GFEI), University of Leeds. The aim of his research is to enable healthy, sustainable diets through identifying and characterizing functional properties of food components, notably proteins and phytochemicals from a wide variety of indigenous and cultivated food sources such as pulses, cereals, pseudo-cereals, algae, insects, etc. A key area is understanding the role of processing on the biological value and safety of proteins and peptides. The long-range goal of Dr. Hernández-Álvarez's research program is to enhance the health of individuals by identifying and determining the benefits of plant foods' bioactive components. His research on legumes, algae, oilseeds, cereals, and insects have led to an increased understanding of the antioxidant, antihypertensive, anti-inflammatory, and other physiological properties of these foods. To date, he has contributed to over 60 publications, book chapters, and conference abstracts in the research areas of protein extraction, bioactive peptides, and food processing.

Martin Mondor is a chemical engineer by training. He received his bachelor's degree (cooperative program) from the Université de Sherbrooke in 1995. He received a doctorate from the Université de Sherbrooke in 2000. In June 2004, he completed a three-year post-doctorate at Agriculture and Agri-Food Canada's Saint-Hyacinthe Research and Development Centre (SHRDC) in Saint-Hyacinthe. He has been a research scientist at the SHRDC since February 2005 and specializes in membrane technology applications in the agri-food industry. Dr. Mondor's work aims to strengthen the sector's competitive position by focusing on value-added production and processing. He collaborated with different Canadian Industry Companies such as Ferme du Petit Chenal, InnoVactiv, Lassonde Industries, among others. Dr. Mondor has authored or co-authored more than 200 scientific contributions in the form of book chapters, scientific articles, research reports, and presentations for scientific conferences. He is an external reviewer for over 20 scientific journals. In 2015, Dr. Mondor received the International Association for Engineering and Food's (IAEF) Distinguished Service Award for his outstanding contribution to the International Conference of Engineering and Food (ICEF) and IAEF activities which included the organization of the ICEF 12 conference held in Quebec City in June 2015. In 2021, Dr. Mondor was part of the team which received the Prize for Outstanding Achievement in Science from the Science and Technology Branch of Agriculture and Agri-Food Canada for their work on Novel technologies for the delivery of antibiotic alternatives in livestock production. He was also part of the team on the Climate Change Roadmap that received the Deputy Minister Commendation Award in 2022.

Matthew G. Nosworthy received his Ph.D. in amino acid metabolism from the Department of Biochemistry at the Memorial University of Newfoundland in 2015 where he studied di−/tri-peptide transport in the Yucatan miniature piglet during growth and in states of disease. His three-year postdoctoral fellowship at the University of Manitoba from 2015 to 2018 investigated the effect of processing methods on in vivo and in vitro measures of plant protein quality. Dr. Nosworthy then accepted a research associate position in the College of Pharmacy and Nutrition at the University of Saskatchewan where he examined the relationship between genetic background and nutritional composition of pulse crops, how soil quality in Ethiopia alters crop nutrient content, as well as shelf-life and nutritional composition of extruded plant-based snacks. In April 2020, he accepted a Research Scientist position at the Guelph Research and Development Centre, Agriculture and Agri-food Canada, where he is currently investigating plant-based protein nutrition and metabolism. Dr. Nosworthy's research interests are centered on protein nutrition as related to novel protein sources. This includes the determination of amino acid composition and protein digestibility (protein nutritional quality), investigation of the bioavailability of amino acids/peptides using in vivo and in vitro techniques, as well as the identification of bioactive properties present in proteins post-digestion and how they can benefit human health. He has worked on diverse plant crops

including soy, pulses, canola, cereals, and hemp at various stages of the food supply chain. To date, he has contributed to over 65 publications, book chapters, and conference abstracts in the research areas of amino acid biochemistry and protein nutrition.

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Alkaline Extraction–Isoelectric Precipitation of Plant Proteins

1

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Abstract

Alkaline extraction–isoelectric precipitation has been the most used method to extract protein from plant foods. The sample preparation will depend on the features of the starting material. For instance, in solid samples, the method starts with homogenization until producing a powder with a certain particle size distribution. It is recommended to homogenize the particle size of the starting material to increase reproducibility of the extraction. Subsequently, the proteins are extracted using an alkaline pH far from the isoelectric point of the proteins. The isoelectric point is the pH at which the proteins' net charge is zero. Afterwards, the pH is adjusted to the isoelectric point to promote protein precipitation and isolation. As discussed in this chapter, this method has some advantages and disadvantages that need to be addressed to optimize the extraction. Compared to other methods, it is inexpensive, it has a relatively easy protocol, however, it does not represent an environment-friendly option because of the solvents, wastewater, and toxic reactive species such as lysinoalanine that can form at high pH. Resulting proteins can also be denatured and have poor functional properties. In this chapter, the principles, relevance, and technological implications of this extraction method are discussed.

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Keywords

Protein recovery · Alkaline extraction and isoelectric precipitation · Plant proteins · Protein solubility · Isoelectric point · Structural properties · Alkaline solubilization · Protein isolates

1.1 Introduction

Nowadays, the industrial production of proteins has increased because of their commercial demand for diverse products where the protein recovery process of different sources is a key step in the production process, such as food and biotech industry, among others. The need for an efficient, economic, and feasible method to purify the protein of interest has resulted in numerous protocols developed to obtain the highest protein yield under specific conditions. One of the most useful and traditional methods for protein purification is alkaline extraction–isoelectric precipitation, considered a simplistic approach with a minimum amount of low energy steps representing benefits to the protein industry because it is relatively easy and inexpensive to conduct (Vilg and Undeland 2017).

The selection of the best method to purify and isolate a specific protein(s) depends on different properties such as size, shape, charge, hydrophobicity, and affinity for other molecules (Alberts et al. 2019). Additionally, the nature of the protein source also influences the quality of the final product and the performance of extraction. The alkaline solubilization is a traditional method widely used to extract proteins of any source of industrial interest using alkaline solutions (generally within the pH range of 8–11) such as NaOH at different concentrations, raw material-extraction media ratio, temperatures, and time of treatment (de Souza et al. 2016). Its effectiveness and feasibility have been widely proved and used as a method for extraction of proteins, including for those that are hardly water soluble given that in an alkaline environment proteins with a high hydrophobicity increased their surface charge enhancing their solubility in water (Shen et al. 2008).

Once those proteins are extracted using an alkaline solution, they can be recovered by isoelectric precipitation (Geng et al. 2019) adjusting the pH of the solution to the isoelectric point of the proteins of interest. The main challenge in using this method is finding the pH value to obtain the highest yield on protein extraction, which is influenced by the nature of the protein source, type of alkali, concentration, duration, and temperature of the treatment. All these factors are influenced by the intrinsic (physicochemical properties, protein structure and conformation, amino acid composition, hydrophobicity) and extrinsic (pH, ionic strength, temperature, time taken to interact with and influence of other food components) factors which depend on the raw material used as protein source (Khalid et al. 2003; de Souza et al. 2016). Depending on the protein content obtained after extraction, the resulting ingredients are classified into three groups as protein isolates with >90% protein, protein concentrates with protein content between 65–90%, and protein-rich flours with <65% (Oreopoulou and Tzia 2007; Pojić et al. 2018).

In this chapter, we are summarizing the relevance of the alkaline extraction–isoelectric precipitation as one of the most useful techniques to isolate proteins of industrial interest from different sources, especially those extracted from plants. Also, we present an overview of this method, its principles, its common operating parameters, and the type of equipment available to perform this process, as well as its advantages, limitations, efficiency, and its impact on protein technological functionality and bioactivity.

1.2 Principles of Alkaline Extraction–Isoelectric Precipitation

1.2.1 Generalities

The alkaline solubilization is a traditional method widely used for protein extraction from plant sources where its effectiveness and feasibility has been proved widely (Khalid et al. 2003; de Souza et al. 2016; Vilg and Undeland 2017; Geng et al. 2019; Yadav et al. 2020; Likittrakulwong et al. 2021). The alkaline extraction is especially useful to extract proteins from plant cells as these present low water solubility due to the presence of disulfide bonds between their molecules and their hydrophobic nature. Protein precipitation is the process used to separate proteins from a solution by modifying the protein solubility by changing their environment, for instance, altering the net charge, or adding salts (Meng et al. 2018). To better understand how the alkaline solubilization occurs, it becomes relevant to consider the solubility and classification of different types of proteins.

Although the current protein knowledge may lead to more systematic and scientific classifications (conserved domains, structure, sequence, etc.), Osborne classification is widely applied to plant proteins, where these protein fractions still have a high level of biological and functional significance (e.g., in food processing). However, it is important to consider that this classification depends on the conditions used for the meal preparation or/and seed pretreatment, as well as the way the fractionation is performed (e.g., time of extraction, liquid-to-seed ratio, proportion of alcohol, salt concentration, etc.).

Some of the most common proteins recovered from plant sources are:

- Albumins: these proteins are readily soluble in water. They are rich in lysine and valine. Albumins can also precipitate in the presence of high amounts of salts, a process commonly known as “salting out” or be coagulated by heat (Zheng et al. 2019). Due to their high solubility in water, extracts obtained from most plant tissues comprise mixtures of diverse components, for example, carbohydrates, water-soluble phytochemicals (Hoogenkamp et al. 2017), among others. In seeds, there is a particular type of storage proteins called 2S albumins, which are albumin-type proteins with a sedimentation coefficient around 2 (Souza 2020). The sedimentation coefficient of particles characterizes their sedimentation when using centrifugal force, the greater the value, the faster the molecules sediment (Schuck 2016). Several 2S albumins have inhibitory enzymatic activities such as

α-amylases, serine proteases, as well as antifungal, antimicrobial, and insecticidal activities. Additionally, the allergenicity of several seeds such as peanuts, barley, pistachios, sesame, among others, is attributable to some conserved areas of the 2S albumins (Souza 2020).

- Globulins: these proteins are globular proteins that are insoluble or nearly insoluble in water. However, their solubility is increased by the addition of salts. As it is the case with albumins, globulins coagulate when heated. Globulins are also rich in lysine and valine (Orona-Tamayo et al. 2017). Globulins have been extensively studied and characterized, particularly in nutritionally important legumes and oilseeds. They represent the major storage protein of legumes and oilseeds such as lupin, common bean, pea, soybean, among others. The sedimentation coefficients of globulins range from 7S to 12S (González-Pérez and Arellano 2009). Legumin-like globulins are generally designated by names according to their plant origin as glycinin (soybean), arachin (peanut), helianthinin (sunflower), phaseolin (common bean), among others. Hydrolysis of globulins from different legume sources have been proved to yield bioactive peptides with diverse biological potential (Muñoz et al. 2018; Moreno et al. 2020).
- Prolamins: prolamins are water-insoluble proteins that are soluble in aqueous alcohols solutions (70–80% v/v). They contain high amounts of proline and amide nitrogen, but they are deficient in lysine (Chen et al. 2022). These proteins are rich in methionine and cysteine. Prolamins are the major storage protein in various cereals, except for rice and oats, where glutelins and globulins are the major proteins. Common prolamins are assigned names according to their plant origin, such as hordein (barley), gliadin (wheat), secalin (rye), zein (maize), or avenin (oat). Cereal prolamins are present as monomers or low molecular weight aggregates, while glutelins form large disulfide-bonded aggregates (Neyra 2019; Wang et al. 2021).
- Glutelins: these proteins are insoluble in water and alcohol (either diluted or absolute), but they are soluble in diluted alkaline or acid solutions. As part of their composition, glutelins are rich in leucine, threonine, and histidine (Sánchez-López et al. 2020; Zhu et al. 2021). These proteins are a heterogeneous mixture of diverse polymers bound by disulfide-bond polypeptides. Glutelins are also a group of storage proteins found in plant foods, such as wheat, rye, and barley. In terms of technological functionality, they provide body and texture to various baked products (Honda et al. 2021).

1.2.2 Protein Solubilization in Alkaline Solutions

The main feature in the pH-shift process-based protein solubilization is the exploitation of pH-influence on the solubility of proteins in hydrated and homogenized material. In a common alkaline protein extraction, the dissolved proteins are separated from undissolved material using a solid–liquid separation method such as centrifugation, filtration, and others.

Proteins are macromolecules that can undergo changes in their structural conformation according to changes in their environment. At extreme pH values, strong positive and negative charges, respectively, drive proteins apart by repulsion (Gao et al. 2020). The conformation of a protein is also dependent on the sequence of their amino acids in the amino acid chains that fold to minimize the free energy. As proteins get an overall negative net charge (alkaline media), they gradually develop electrostatic interactions with water molecules, thus increasing their solubility (Alavi et al. 2021). An important feature of proteins subjected to alkaline pH values is that they partly unfold and maintain their primary and partially their secondary structure. A wide number of plant proteins have been extracted using alkaline solubilization such as proteins from legumes, oilseeds, and cereals (Amagliani et al. 2017; Accoroni et al. 2020; Xu et al. 2021). Common pH values of protein solubilization range from 8 to 11. Regarding other macromolecules present in the raw materials, most of the insoluble complex carbohydrates tend to precipitate in aqueous solution and can be removed by further centrifugation. Simple carbohydrates will be solubilized along with the protein at alkaline pH and will remain soluble at the pI of proteins. If the sample contains a significant amount of lipids, a defatting step is recommended as the lipid molecules can undergo saponification at alkaline pH, interfering with the protein extraction (Gerde et al. 2013; Muñoz et al. 2018; Lorenzo-Hernando et al. 2019; Wen et al. 2022).

1.2.3 Isoelectric Protein Precipitation

Upon protein solubilization using alkaline extraction, a precipitation method is needed to recover the dissolved proteins. In that sense, the isoelectric point (pI) of proteins is a physicochemical principle that can be utilized to precipitate dissolved proteins in alkaline solutions. The pI represents the pH in a solution at which the net charge of a protein becomes neutral (zero). At pH values above the pI, the proteins' surface is predominantly negatively charged, and therefore, negatively charged molecules will promote repulsive forces (Mohanta et al. 2019). In a similar manner, at pH values below the pI, the surface of the protein is predominantly charged in a positive manner, and in an alkaline solution, repulsion between proteins happens. When adjusting the pH at the pI value, the negative and positive charges are balanced, reducing the repulsive forces (mainly electrostatic), and the attraction forces among proteins predominate, causing complex formation, aggregation, and precipitation (Fig. 1.1) (Kozlowski 2017; Pergande and Cologna 2017). At the end of the alkaline extraction, a complex protein mixture of proteins is obtained. The isoelectric point of a protein depends on the nature of the different ionizable groups present in its surface. Therefore, considering that each protein presents a specific pI, there is not only one pI for the mixture. It is necessary to determine the pI of the proteins of interest prior to the separation step (Yadav et al. 2020).

The pI of most plant proteins is in the pH range of 4–6 (Pergande and Cologna 2017). After isoelectric precipitation, a centrifugation step allows the recovery of precipitated proteins to be used for further experiments. In summary, the principle of

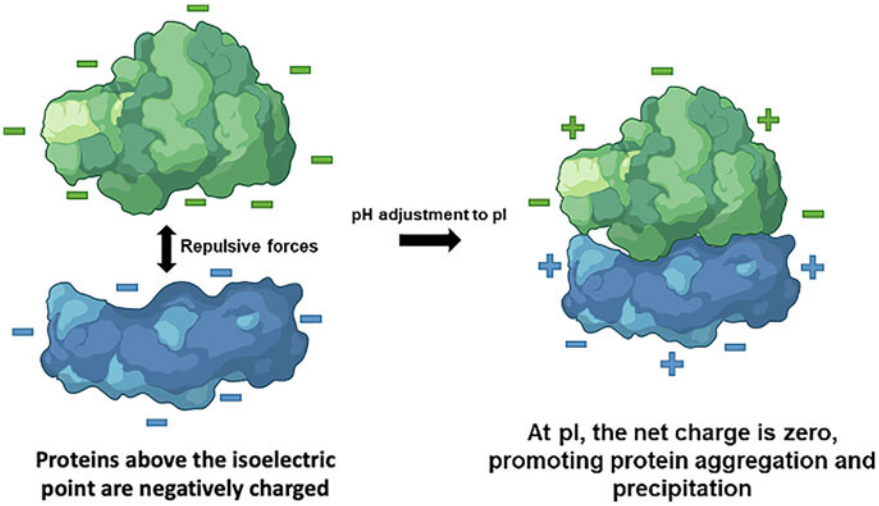

Fig. 1.1 Diagram exemplifying the isoelectric precipitation principle

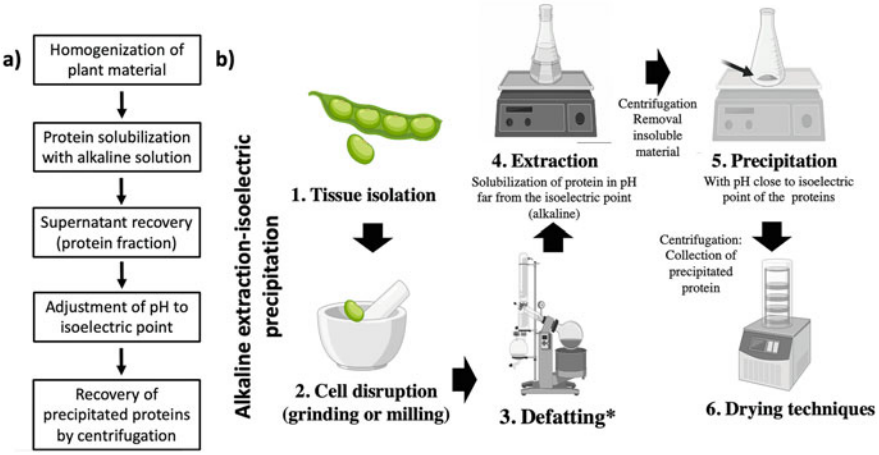

Fig. 1.2 Diagrams showing (**a**) a general alkaline–isoelectric precipitation extraction method for plant proteins, and (**b**) laboratory procedure of alkaline–isoelectric precipitation. *This step is optional depending on the protein source

the alkaline-isoelectric precipitation is based on the solubilization of the proteins at alkaline pH. Afterwards, the pH is adjusted to the pI, and therefore, most of the proteins precipitate. Precipitated proteins are then recovered by centrifugation. In case of proteins for food applications, the precipitate requires a water resolubilization step and neutralization prior to drying (Fig. 1.2).

1.3 Common Operating Parameters and Types of Equipment Available

1.3.1 General Considerations and Sample Preparation

To achieve high efficiency in the protein extraction, it becomes necessary to characterize and optimize the extraction of proteins from the specific biological material, since the release of proteins to the extracting solution can be hindered by different components of the material. The main variables that can be analyzed are temperature and size of particle. Firstly, in the case of temperature, it is important to maintain a low temperature along all the process of extraction to avoid protein denaturation. Most of the proteins are thermolabile, additionally, temperatures around 30 °C maximize the activity of a great variety of plant enzymes. Biological materials present a high quantity of endogenous proteolytic enzymes, and their activity is not desirable when proteins are the product of interest. Although a temperature around 4 °C is preferred to avoid protein denaturation, in some cases high temperature increases the yield of extraction of proteins (Oliyaei et al. 2017). Besides considering an optimal temperature to increase the protein extraction efficiency, it is important to take in consideration that some functional properties can be affected when high temperatures are used. Therefore, the setting of the optimal extraction temperature must consider the final use of the proteins (protein-rich flours, bioactive peptide generation, enzyme recovery, etc.) (Deak and Johnson 2007; Moore et al. 2021).

Secondly, in the case of particle size, typically the external tissues of biological materials are composed by different types of fibers that act as barriers. In some cases, such as vegetal materials, structural components of the cell wall impede the release of proteins into the extracting solution (Fleurence et al. 1995). Therefore, grinding and milling of the material is required to break down these structural components of biological materials. The particle size obtained from this pretreatment affects the extraction of proteins. Usually, a higher protein extraction yield is obtained when small particles are extracted. This is because, for small particles, a higher surface area is in direct contact with the solvent than for large particles. However, in some cases depending on the type of material, the protein extraction yield may present a U-shape behavior due to high solubility profile at low and high pH, and loss of favorable water–protein interactions close to protein pI (Vishwanathan et al. 2011). In that case, using the smallest particle size could not result in optimal extraction yield. In starting materials rich in fiber, when the smallest particle size is used, a higher quantity of fibers may be exposed to water increasing their water-holding capacity and affecting the extraction of proteins (Yamazaki et al. 2005). Some carbohydrate-degrading enzymes can be used to increase the extraction efficiency through the degradation of structural components of the cell wall and complex carbohydrates, which increases the release of proteins into solution, depending on the level of fiber content from the original material (Vilg and Undeland 2017). Therefore, a characterization of the effect of temperature and particle size is

suggested to increase the efficiency of extraction of proteins from different biological materials.

To simplify homogenization (manipulation of the sample, avoidance of undesirable biochemical changes in the material, loss of material, etc.), the sample can be freeze-dried or only frozen before this step. Several procedures can be used to dry the material at lab scale, lyophilization or a dryer are some options. Briefly, lyophilization involves pre-freezing (up to 1 h, from room temperature to −40 °C), primary drying (about 10–20 h, stepwise temperature and pressure change up to ~30 °C and 1013 hPa, respectively), and secondary drying (3–10 h, up to 40 °C), using sublimation as the main physical phenomena. Drying procedures on several lyophilized biological samples have shown that storage at 4 °C for 20 months does not impact the quality and quantity of potentially extractable proteins (Molnar et al. 2021).

As mentioned before, when the plant materials have a significant content of lipids, lipids are usually removed to increase the protein content in the extract and to avoid coprecipitation. In general, for plant materials with a lipid content less than 5% no defatting process is needed, for instance lentils or beans; while chia seeds (≈30% lipids), soybean (≈18% lipids), or canola (≈35%) require a defatting pretreatment to optimize protein extraction (Fetzer et al. 2018). One of the most common defatting treatments is using highly non-polar solvents such as hexane or petroleum ether. This method is widely used at lab scale, but environmental impact, health hazards, and cost should be considered when the process is scaled up. The extraction of fat can be achieved using Soxhlet apparatus, percolation, maceration, or washing several times. At industrial scale, cold pressing is used to partially defat biological materials using mechanical pressure. Once the material has the appropriate particle size and is defatted, it is ready to be used for protein alkaline extraction and isoelectric precipitation (Luna Vital et al. 2014; Xing et al. 2018; Grancieri et al. 2019; Moreno et al. 2020). Another defatting method widely used at laboratory and industrial scale is supercritical CO_2 lipid extraction. The principle behind this method is that a fluid (CO_2) is brought to a particular combination of pressure and temperature that allows to obtain supercritical properties for the selective lipid extraction from the starting materials.

1.3.2 Operating Conditions

The main operating conditions of alkaline extraction of proteins from biological materials are pH and temperature. Proteins are more soluble under alkaline conditions since charged and polar groups of proteins are more exposed to water molecules of the solvent (Kristinsson et al. 2006). The pH-dependent solubility of proteins can be influenced by protein–protein, protein–solvent interactions, and surface hydrophobic-hydrophilic balance of proteins (Horax et al. 2011). In the case of temperature, as mentioned before, a low temperature is suggested to avoid denaturation. Depending on the use of the protein extract, in some cases, high temperatures are used considering partial or full denaturation of proteins and effects on functional properties of the protein extract. For instance, when the application of

Table 1.1 Isoelectric point of proteins from different biological materials

Source	Isoelectric point (pI)	Reference
Soybean (*Glycine max*)	4–5	Rickert et al. (2004)
Almond (*Prunus dulcis*)	4.5–5.5	Li and He (2004)
Common bean (*Phaseolus vulgaris* L.)	4.7–5.0	Li and He (2004)
Wheat gluten	6.2	Liu et al. (2013)
Pea (*Pisum sativum*)	4.5	Barac et al. (2010)
Faba bean (*Vicia faba*)	5.0–5.5	Gundogan and Can Karaca (2020)
Amaranth (*Amaranthus hypocondriacus*)	4.0	Figueroa-González et al. (2022)
Chickpea (*Cicer arietinum* L)	4–6	Boukid (2021)
Hemp (*Cannabis sativa* L.)	4.3	Rodriguez-Martin et al. (2020)
Lentil (*Lens culinaris* L.)	5.2	Lee et al. (2021)
Lupin (*Lupinus angustifolius*)	4.0–6.0	Rodríguez-Ambriz et al. (2005)
Seaweed (*Saccharina latissimi)*	<3	Vilg and Undeland (2017)
Blue-green algae (*Spirulina platensis*)	3.0	Devi et al. (1981)
*Scenedesmus acutus (*microalgae*)*	3.5	Venkataraman and Shivashankar (1979)
Tetraselmis sp. (green microalgae)	4.0	Schwenzfeier et al. (2011)
Nannochloropsis oculata (microalgae)	3	Cavonius et al. (2015)

the extracted proteins is aimed to enzyme recovery or proteins with a native structure are needed, low temperatures will be more suitable. On the other hand, a thermal pretreatment of proteins improves enzymatic hydrolysis and in the case of food applications, it also improves some techno-functional properties such as gelation (Boyle et al. 2018; Gao et al. 2020; Yang et al. 2021). Other operating conditions are agitation speed (varies depending on the equipment used) (Perović et al. 2020), solubilization time (1 h to overnight) (Yang et al. 2021), and w/v ratio that varies from 1:4 to 1:15.

After protein extraction, isoelectric precipitation is carried out to recover protein isolates. In this case, the solubility of proteins is decreased using a pH-shift from alkaline conditions to acid conditions. The pH used to precipitate proteins is usually near to the isoelectric point (below 6). In Table 1.1, isoelectric points of proteins from different biological materials are presented. Also, a low temperature (around 4 °C) is usually used during this process to increase the yield of precipitation.

Additionally, the precipitation yield could be improved by combining different methods to precipitate proteins such as increasing the ionic strength (adding some salts) or adding flocculating/precipitating agents (Burgess 2009; Yoshikawa et al. 2012; Wingfield 2016).

1.4 Efficiency and Impact of Isoelectric Precipitation from Different Plant Sources

As animal proteins are usually linked to negative environmental impact, research focusing on plant proteins has been increasingly prioritized, aiming to obtain suitable protein-derived products with functional and biological applications. Moreover, plant-based proteins are cholesterol-free, abundant and exhibit lower saturated fatty acids than their animal-origin counterparts (Gençdağ et al. 2021).

Isoelectric precipitation can be successfully used to extract underutilized and sustainable proteins from several plant sources. For instance, Vogelsang-O'Dwyer et al. (2020) reported the feasibility of using dry fractionation and isoelectric precipitation of proteins from dehulled Faba bean (*Vicia faba*). To achieve this, an aqueous-extracted faba bean isolate was produced from a single-batch pilot-scale processing. The obtained isolate exhibited a higher protein (90.1 g/100 g vs. 64.10 g/100 g), fat (4.36 g/100 g vs. 2.43 g/100 g), but lower total carbohydrates (0.34 vs. 28.7 g/100 g) content than dry-fractioned protein. For the protein profile, the authors reported visible bands containing most proteins reported in pulses such as convicilin, legumin, vicilin, α-legumin, and β-legumin. Scanning electron images (SEM) indicated a major presence of discrete particles (particles with a rigid and spherical shape) for the isolate obtained by isoelectric precipitation, as well as similar particle size distributions, and lower hydrophobicity than dry-fractioned proteins. The isolate also showed the lowest foaming properties, fat absorption capacities, and a higher concentration of protein was required to obtain a gel (12% protein vs. 7% protein from dry-fractioned proteins), leading to weaker gel structures for a given concentration. Nutritionally, the isoelectric procedure slightly affected the amino acid profile and showed higher removal of antinutritional components, potentially proper for low fermentable oligo-, di-, and monosaccharides and polyols (FODMAP) formulations.

Specific protein fractions can be obtained from alkaline solubilization and isoelectric precipitation of pea (*Pisum sativum*) protein, starting from pea flour soaking in water (pH 8.0), centrifugation, protein precipitation from supernatants at pH 4.5, and separation into two fractions: globulin-rich fraction (GLB-RF, from re-dispersion at pH 7.0 and freeze-drying) and albumin-rich fraction (ALB-RF, from 2 kDa diafiltration and freeze-drying) (Kornet et al. 2022). While the pea protein concentrate contained 54.8% protein, ALB-RF displayed 52% protein, and GLB-RF 86.3%. Examination of their thermal properties showed that most proteins from the fraction were native and exhibited denaturation temperatures close to reported values for pea-derived proteins (75–85 °C). GLB-RF fractions (0.7% w/w) were more viscous (1.09 mPa·s) than ALB-RF fractions (1.07 mPa·s). Analyses of Langmuir-Blodgett films made from fractions indicated that GLB-RF-based films could be stabilized at a surface pressure of 15 mN/m, while ALB-RF-based films did not present this property. However, ALB-RF showed the highest overrun (up to 250%) and half-life time (up to 300 min) foams.

Reverse micelles technology as a novel and mild extraction procedure proteins was compared to alkaline extraction and isoelectric precipitation of walnut (*Juglans*

regia L.) proteins (Zhao et al. 2019). After defatting, walnut powders were dispersed in NaOH solution (pH 9.0) at 1:5 ratio and stirred (1 h, 45 °C). After centrifugation (22,470 × g, 20 °C, 20 min), the solutions were dialyzed (4 °C, 24 h) to remove impurities, and several fractions were obtained depending on the protein type (albumin, globulin, prolamin, and glutelin). FTIR analysis confirmed the purity of each fraction by identifying the corresponding peaks of each protein class within the amide I region (1700–1600 cm^{-1}) spectra. Scanning electron microscopy (SEM) images indicated that globulins extracted by both reverse micelles and alkaline extraction showed the clearer structures with the smallest pores, attributed to the ability of alkaline extraction to destroy the protein structure. A similar trend was also observed for the other protein classes, but no additional physicochemical or functional properties were explored by the authors.

Since solubility is a key functional property from protein-derived products, the impact of isoelectric precipitation has also been studied in this matter. Proteins extracted from rapeseed (*Brassica napus* subsp. napus) meal (5% ethanol suspension, pH 12, 40 °C for 60 min) were used to prepare two kinds of protein concentrates: sequential precipitation of the proteins (from pH: 10.5) and lowering the pH by 1 unit to 2.5, using HCl; the other one consisted in pH elevation from 2.5 to 8.5 (Kalaydzhiev et al. 2020). The precipitates at each final pH value were obtained after centrifugation (18,000× g, 15 min) and the supernatant was subjected to isoelectric precipitation. Pooled precipitated proteins at each variation of 1 pH unit were also obtained. Both protein concentrates contained up to 72.84% total crude protein, 10.45–13.18% ash, and up to 0.71% of total polyphenols (Folin-Ciocalteu method). The electrophoretic analysis of both isolates showed a noticeable low molecular weight protein group (up to 50 kDa), and the authors hypothesized the presence of 2S-group albumin in the samples. High amino acids scores were found for the isolates with valine (152.56%), leucine (114.23%), and isoleucine (75%) showing the highest values. For the non-essential amino acids, glutamate, alanine, and proline were the most represented. Regarding solubility, isolates were highly soluble (>60%) at pH >6.5, suggesting the ability of sequential precipitation for the preparation of proteins with enhanced functional properties.

The way extraction is conducted could affect the enzymatic activity and their aromatic profile. Gao et al. (2020) studied the impact of alkaline extraction on the structure and techno-functional properties of yellow pea protein concentrates, with a particular focus on its beany flavor after lipoxygenase activity. The authors performed alkaline extraction–isoelectric precipitation mixing the pea flours with water (1:15 w/w ratio), adjusting pH (8.5, 9.0, and 9.5), stirring, centrifuging, and adjusting the pH of the supernatants until a value of 4.5 was reached. Collected precipitates were then pH adjusted to 7.0, and resulting powders were freeze-dried for 48 h. Extraction yields up to 15.36% and > 80% crude protein (84.67, 83.33, and 83.40% at pH 8.5, 9.0, and 9.5, respectively) were obtained, agreeing with the enhanced total negative charge of solubility of proteins after basic amino acids neutralization. However, increasing pH reduces protein solubility (~80, 88, and 93%, respectively, for pH 8.5, 9.0, and 9.5) as the higher pH promotes protein aggregation. The identification of volatile compounds linked to the alkaline

extraction indicated that pH 9.5 increased the amounts of 1-octen-3-ol compared to the other pH-assisted extractions (+63.63% and + 28.57% when compared to pH 8.0 and 9.0, respectively) from lipoxygenase activity over lipids (beany flavors), while the flavor profile of pH 8.5 and 9.0 was lower than the original flour. Hence, the authors suggested pH 9.0 as the optimal value to obtain functional, protein-rich isolates with low beany flavor.

Combining isoelectric precipitation with other technologies can improve protein properties with a low carbon footprint. Hadidi et al. (2020) suggested the use of ultrasound and ultrafiltration with assisted isoelectric precipitation (UUAAIP) to produce alfalfa (*Medicago sativa*) protein isolate for human consumption. This technique is based on alfalfa leaves blanching (1/10 w/w leaves:steam), defatting (hexane, 25 °C), dispersion in water in ultrasonic cleaning bath, pH adjustment (9–11), and extraction at 30-50 °C with ultrasonic temperature control. A peristaltic pump recirculated the solution to a feed tank through a 10 kDa molecular weight cut-off ceramic membrane. The obtained precipitates from the filtration retentate were centrifuged, acidified (pH 3.4), neutralized, and freeze-dried. Optimized conditions showed a solvent/material ratio of 43.3 mg/L, pH 10.1 for protein extraction at 42.5 °C and 102 min. This method (UUAAIP) showed an extraction yield of 14.5% compared to the other treatments (13.90% and 16.60% for heat-coagulation—HCE and alkaline-isoelectric precipitation extraction methods—AIPE, respectively), but higher protein contents (UUAAIP: 91.1%, AIPE: 74.5%, HCE: 63.9%). Moreover, saponin content was reduced up to 3.92-fold, while polyphenols content was reduced by ~50%. Produced isolates (UUAAIP and alkaline-isoelectric precipitation extraction) displayed the highest solubility values (>75%) at pH > 8. The authors conclude that UUAAIP enhances the average molecular weight, color, and protein content of extracted isolates.

A combination of phytase treatment with alkaline solubilization and isoelectric precipitation of seabuckthorn (*Hippophae rhamnoides* L.) seed protein showed changes in the potential protein functionalities of this food source (Xiang et al. 2022). The defatted flour was ground and screened through a 100-mesh sieve and dispersed in NaOH (0.1 M, 3600× g), incubated, and shook. The mixture was then centrifuged, and the collected supernatant was pH-adjusted to 5.0. The product was either treated or not with food-grade phytase from *Aspergillus niger* and divided into 4 portions (50 °C for 1, 2, 3, and 6 h, respectively). The longer the final incubation, the lighter and yellower the solutions were. After 3 h-phytase treatment, the total protein significantly increased, but these treatments decreased total phenolic, total flavonoids, and total proanthocyanidins, although the absence of phytase did not prevent this trend but the effect was larger for phytase-treated samples. An in vitro digestibility procedure assayed for all the samples indicated a more digestible 3 h phytase-treated sample than its non-phytase-treated counterpart, explained by improved protein solubility due to phytase challenging and the elimination of phytic acid, a known pepsin activity inhibitor.

Multi-enzymatic approaches, followed alkaline precipitation, were applied to obtain barley (*Hordeum vulgare* L.) protein concentrates (Houde et al. 2018). Defatted powders were extracted with 0.5 M NaOH (pH: 11) for 2 h at 23 °C,

centrifuged, and the supernatant was dialyzed for 48 h at 4 °C and freeze-dried. For the sequential precipitation, the powders were subjected to pH adjustment (4.5) instead of dialysis, left precipitate overnight (4 °C), centrifuged, and precipitates were re-suspended in water, dialyzed, and freeze-dried. For the enzymatic treatments, digestion with α-amylase, α-amylase + amyloglucosidase, and α-amylase + amyloglucosidase + β-glucanase were conducted. Isoelectric precipitated proteins yielded the highest amount (~70%), but tri-enzymatic-treated powders exhibited the highest protein recovery yields (~75%). The alkaline procedure was mainly governed by low molecular weight fractions (<20 kDa) (globulin and albumin). Proteins at pH 3.0 showed the highest overall foaming capacities (60-80%), and the highest foam stability was obtained for pH 5.0. The authors concluded that enzymatic treatments allow differential functional properties that could be further explored in practical food applications.

Alkaline extraction was used to obtain hempseed (*Cannabis sativa*) meal protein isolates (Hadnađev et al. 2018). Ground and defatted meal powders were subjected to isoelectric precipitation after water suspension, pH adjustment to 10.0 by 1 M NaOH under constant stirring, and centrifugation. The collected supernatant was pH adjusted (5.0) and left overnight at 4 °C to allow precipitation, followed by centrifugation, washing, and re-suspension in water. Results for several physicochemical properties of the alkaline-precipitated proteins were compared with micellization, an extraction technique dispersing the defatted powder in NaCl, centrifugation, and ultrafiltration. Isoelectric precipitation resulted in a higher protein yield (up to 50.60%, based on protein weight) but obtained powders contained less protein (91.44% vs. 98.87%) than micellization. Isoelectric precipitation-powders displayed more total phenolic compounds (up to 139.89 mg gallic acid equivalents, GAE/100 g fresh weight), and both procedures favored globulin rather than albumin extraction. Both techniques exhibited a similar amino acid profile, particularly for arginine, aspartic acid, and glutamic acid. Isoelectric precipitation-obtained powders displayed the lowest solubilities at pH: 6.0, a higher enthalpy, and denaturation temperature values, but higher thermal stability and structural order were shown in the micellization-obtained powders. A lower water-holding capacity was exhibited for the micellization-resulting powders, while there were no changes in the fat absorption capacities.

Examination of the physicochemical and functional properties of red lentil (*Lens culinaris*) proteins was conducted at several pH extractions (Lee et al. 2021). Commercial dehulled red lentils from different origins (USA, Nepal, and Turkey) were powdered (100-mesh sieving), dispersed in ionized water, pH-adjusted (9.0), homogenized (150 rpm, 30 min, room temperature), centrifuged, pH-adjusted (4.60), and precipitates were centrifuged again and pH-adjusted (7.4). Samples from Nepal showed the highest total amino acid content (74.84 g/100 g red lentil protein), followed by the USA (69.62 g/100 g red lentil protein) and Turkey (67.24 g/100 g red lentil protein). The three extracted protein concentrates showed a similar solubility and zeta potential trend, whereas Turkey- and Nepal-origin red lentil proteins displayed the highest oil absorption capacities (7.5–8.0 g/g red lentil protein). For all

the extracted proteins, the highest foaming capacities were obtained at pH 2.0, while the best foam stability index was obtained at pH 6–7.

Alonso-Miravalles et al. (2019) investigated the technological features of sustainable protein isolate from lentils obtained through membrane filtration and isoelectric precipitation. High molecular weight proteins were extracted, subjecting lentil flours to pH 7.5, while isolates were obtained after acid precipitation at pH 4.5, neutralization (3 M NaOH), pasteurization (65 °C, 30 min), and spray-drying. The obtained isolates with fixed conditions, without conducting an optimization, showed high protein content (85.13–93.7%) and exhibited a common pulses protein profile (convicilin, vicilin, and α- and β-legumins). Little conformational changes were found for the secondary structure of the extracted isolates and heterogeneous rounded particles (10–50 μm) were observed. High surface hydrophobicity was found (2688), higher than reported values for legumin-like proteins (2000). Combined results from isolates displayed that both reached high solubilities (40-50%) and the low zeta potentials (−25 to −30 mV) at pH >7.0, water-holding capacities among the average (2.60–3.96 g/g) compared to several legumes, and a quarter of the environmental impact from producing caseinate or whey. The estimation was carried out as life cycle assessment using Umberto 5.5 software, considering several factors such as aquatic and terrestrial eutrophication, acidification, photochemical oxidants formation, particulate matter, stratospheric ozone depletion, use of phosphorus, and land use, among other. Alkaline extraction–isoelectric precipitation has also been used to isolate proteins that are further processed through enzymatic hydrolysis to generate bioactive peptides. Different legumes such as lentil, black bean, chickpea, among others, were used to extract proteins by alkaline extraction–isoelectric precipitation and through a gastrointestinal digestion simulation using pepsin-pancreatin, peptides below 10 kDa were generated. The peptides generated were reported to exert enzymatic inhibition in markers related to obesity and type-2-diabetes (Moreno et al. 2020; Chandrasekaran et al. 2020).

Representative studies involving alkaline extraction of proteins and its effect on their properties are summarized in Table 1.2.

1.5 Relevance, Advantages, and Limitations in Alkaline Extraction–Isoelectric Precipitation

1.5.1 Relevance of Protein Extraction in a Global Health Context

One of the main objectives of the plant protein extraction by alkaline extraction–isoelectric point is the development of functional ingredients with technological applications, justified in the need for greater food quantity and quality as worldwide population increases (Pojić et al. 2018). On the other hand, the appearance of emerging pandemics such as the current COVID-19 confirms that an adequate diet helps preventing and reducing the likelihood of several diseases. These are the two crucial reasons that lead to the search for sustainable alternatives to produce functional foods. According to the WHO, nutrition in adults includes a daily diet of

Table 1.2 Protein concentrates and isolates produced by isoelectric precipitation from several plant sources

Plant Source	Manufacturing Procedure	Main Outcomes	References
Faba bean (*Vicia faba*)	Dehulled faba beans were subjected to acidic-wet milling, fiber and insoluble proteins removal, starch separation, and isoelectric precipitation at pH: 4.8 and adjustment at pH: 6.8.	High protein content (90.1%), particle size mainly at 90 μm (44.5%), high solubility at pH > 6, low fat absorption (87.2 g/100 g), 12% protein required for gelation, most essential amino acids are retained (mainly Leucine, Lysine, and Valine). Isolates were more digestible ($p < 0.05$) than dry-fractioned proteins. Isoelectric precipitation significantly removed vicine and convicine.	Vogelsang-O'Dwyer et al. (2020)
Rapeseed (*Brassica napus* subsp. napus)	Proteins extracted with ethanol (5% w/v) were adjusted to pH 12 at 40 °C for 60 min under agitation. Two isolates were prepared as follows: Sequential precipitation starting at pH 10.5 and pH lowering up to 2.5 with HCl; pH increase from 2.5 to 8.5. Both isolates were centrifuged (15 min, 1800× g). Then, pH was adjusted to 8.5.	Protein content: 68.87–72.84%; LMW: 77.8–81.8%, MMW: 9.1–11.1%, HMW: 9.1–11.1%; Main essential amino acids: valine (AAS: 152.56%), Leucine (AAS: 114.23%), isoleucine (75%). Solubility >60% at pH > 6.0-6.5	Kalaydzhiev et al. (2020)
Hempseed (*Cannabis sativa*)	Defatted hempseed meal was suspended in water (1:20 ratio), and the pH was adjusted to 10 using 1 M NaOH under stirring (2 h, 35 °C). The solution was left precipitate overnight a 4 °C, centrifuged (7500 × g, 20 min), washed three times with water to remove salts, and the precipitate was re-suspended in water (pH-adjusted to 7.0). Suspension was freeze-dried and stored at −20 °C. For the micellization, the defatted powder was dispersed in NaCl (1:10	Isoelectric-obtained protein showed a higher protein yield (+25.96%) but contained less protein (−7.51%) than micellization-obtained powders. The isoelectric precipitation-obtained powders were darker, redder, and yellower, but contained more total phenolic compounds (139.89 vs. 101.28 mg GAE/100 g FW). Electrophoretic profiles indicated a major preference for globulin extraction rather than albumin using both	Hadnađev et al. (2018)

(continued)

Table 1.2 (continued)

Plant Source	Manufacturing Procedure	Main Outcomes	References
	ratio), stirred (35 °C, 2 h), centrifuged (6000× g, 20 min), dialyzed against water (4 °C, 72 h) using ultrafiltration (MW cut-off: 12000-14,000 Da), and precipitated proteins were recovered by centrifugation (7500× g, 20 min).	methods, no differences in the amino acid profile were found, and isoelectric precipitation-obtained powders showed less enthalpy (11.77 mJ/mg) and peak denaturation temperature (93.47 °C) compared to micellization-obtained powders.	
Alfalfa (*Medicago sativa*)	Alfalfa leaves were bleached (4.4 min under steam at atmospheric pressure) and 1/10 w/w steam:leaves ratio. Dried leaves were ground (50 mesh size), defatted with hexane (25 °C), dispersed in water (1:30-1: 50 g/mL) in an ultrasonic bath (20 kHz, 100 W). The pH was then adjusted (9-11) with 1 M NaOH, and extraction temperatures were set at 30-50 °C. Retentates were centrifuged (1000 g, 20 min), and collected proteins were acidified until pH 3.5. Washed pellets were adjusted to pH 7.0, and freeze-dried.	Optimized protein extraction was found at solvent/solid material ratio of 43.3 mg/L, pH: 10.1, and temperature: 42.5 °C. the obtained isolates presented protein contents of 91.1%, extraction yields up to 14.5%, 3.92-fold less saponins than conventional alkaline-isoelectric precipitation extraction, but lower ($p < 0.05$) foam capacity (365.8%) and foam stability (53.6%).	Hadidi et al. (2020)
Seabuckthorn (*Hippophae rhamnoides* L.) seed	The defatted powder was ground for 15 s and screened through a 100-mesh and dispersed in a NaOH solution (0.1 M), incubated (37 °C), and centrifuged (5000 × g) at 10 °C for 20 min. Food-grade phytase from *Aspergillus niger* (45 U/kg) was either added or not, and four portions were incubated at 50 °C (1, 2, 3, and 6 h). The portions were centrifuged (5000 × g, 10 °C, 20 min).	Treatments with phytase showed the highest lightness (L*: 49.83–53.31), redness (a*: 9.91–10.8), and the lowest yellowness (b*: 12-72–14.69) values. The phytase treatment increased ($p < 0.05$) protein content compared with those without phytase (~4–5%, up to 650-680 g/kg flour), decreased phytic acid content (−30-32%), but decreased total phenolic compounds (up to −60%), total flavonoids (up to −23%), and total	Xiang et al. (2022)

(continued)

Table 1.2 (continued)

Plant Source	Manufacturing Procedure	Main Outcomes	References
		proanthocyanidins (up to −55%). Protein solubility was the highest between pH 4 and 6 for both phytase and non-phytase treatments. 3 h-non-phytase samples subjected to gastric digestion were the most digestible samples.	
Barley (*Hordeum vulgare* L.)	Defatted flours with hexane (1:10 solvent:flour ratio, stirring: 200 rpm, 1 h, 25 °C) were treated for alkaline extraction or alkaline extraction/isoelectric precipitation and enzymatic treatments. The alkaline extraction was conducted with NaOH (0.5 M, pH: 11, in a 10:1 solvent:flour ratio) for 2 h at 23 °C. The mixture was centrifuged (4000 × g, 15 min), and supernatant was dialyzed (48 h, 4 °C) against water in 2000-Da benzoylated dialysis tubes. For the sequential precipitation, after the alkaline centrifugation, the supernatant was pH adjusted to 4.5 with 0.5 M HCl, left to precipitate overnight (4 °C), centrifuged (4000× g, 20 min), and the pellet was re-suspended in water, dialyzed, and freeze-dried. The enzymatic treatment was conducted using α-amylase, α-amylase + amyloglucosidase, and α-amylase + amyloglucosidase, and β-glucanase.	Alkaline extraction/ isoelectric precipitated proteins exhibited the highest protein contents (up to 70%) and purification factors (protein content initial flour/protein content in the extract >90%) but tri-enzymatic treated alkaline extraction and isoelectric precipitated proteins showed the highest recovery yield (~75%). The alkaline method was dominated by LMW proteins (<20 kDa), whereas fractions of HMW (>85 kDa) proteins were higher in the enzymatic-assisted procedures, compared to the other assayed treatments. The highest foaming capacities (65-90%) were shown at pH 3.0, whereas at pH 5.0 the highest foaming stabilities (50 to >90%) were obtained.	Houde et al. (2018)
Pea (*Pisum sativum* L.)	Alkaline extraction–isoelectric precipitation: Dehulled yellow pea flour (250 mm particle size) was	Extraction yield: 15.36%. Total crude protein >80% at pH 9.5. Extraction at pH 9.0 produced a	Gao et al. (2020)

(continued)

Table 1.2 (continued)

Plant Source	Manufacturing Procedure	Main Outcomes	References
	dispersed in water (1:15 w/w water:flour), pH (8.5, 9.0, and 9.5) was adjusted with 1 M NaOH. The solution was stirred (600 rpm, 60 min), centrifuged (6000 rpm, 20 min), supernatant was filtered (Whatman paper N. 1) and supernatants pH adjusted (4.5) to precipitate proteins. Precipitates were collected, pH re-adjusted (7.0), and freeze-dried (48 h).	balanced extraction yield between protein amount, and low production of beany flavors linked to 1-pentanol and 1-octen-3-ol.	
	Pea powder was soaked in alkaline water (pH 8.9) and protein was precipitated from supernatant after centrifugation at pH 4.5. The globulin-rich fraction (GLB-RF) was obtained from re-dispersion at pH 7.0 and freeze-drying. The albumin-rich fraction (ALB-RF) was acquired from 2 kDa diafiltration and freeze-drying.	Protein contents: 52% (ALB-RF) and 86.3% (GLB-RF). Viscosity (0.7% w/w): 1.09 mPa·s (GLB-RF) and 1.07 mPa·s (ALB-RF). Foaming properties: Overrun (ALB-RF: ~250%; GLB-RF: ~50%); average bubble size (GLB-RF: ~0.25 mm; ALB-RF: 0.05 mm); half-time (ALB-RF: ~300 min; GLB-RF: ~100 min)	Kornet et al. (2022)
Lentils (*Lens culinaris*)	Dehulled red lentil seeds from the USA, Nepal, and Turkey were grinded, screened through a 100-mesh, dispersed in ionized water, pH-adjusted (9.0), centrifuged (12,857x g, 5 min), and supernatants were pH-adjusted until 4.6. Precipitated protein was recovered by centrifugation (12,857 × g, 5 min), washed twice and pH-adjusted (7.4)	Amino acid content: 74.84 g/100 g protein (Nepal), 69.62 g/100 g protein (USA), and 67.24 g/100 g protein (Turkey). Highest foam capacities at pH 2.0. Best foam stability index at pH 6–7	Lee et al. (2021)
	Dehulled lentils were milled until a D50: 21 μm was obtained, and flours were re-suspended in water (ratio not specified) (pH 7.5 for HMW proteins). Insoluble fiber and starch	Protein content: 85.13%, electrophoretic profile containing convicilin, vicilin, α- and β-legumins. Highest solubility values at pH > 7.0. Up to 57.2% foam capacity at 3% w/v	Alonso-Miravalles et al. (2019)

(continued)

Table 1.2 (continued)

Plant Source	Manufacturing Procedure	Main Outcomes	References
	were separated by decanting. For LMW, an isoelectric precipitation was carried out by acid precipitation at pH 4.5. The isolates were neutralized (3 M NaOH), pasteurized (65 °C, 30 min), and spray-dried (IAT: 180 °C, OAT: 75 °C).	content. Lower environmental impact than whey protein isolate production.	
Chickpea (*Cicer arietinum*)	Kabuli chickpea seed powder was adjusted to pH 11.5, 1:15 water:flour, and after precipitated using pH 5.5-6.5. Protein isolates were digested using pepsin and pancreatin. The resulting peptides were sequenced and evaluated for DPPIV inhibitory potential.	An isolate of 75% protein content generated peptides with up to 15 amino acids. Several peptides inhibited DPPIV enzymatic activity, known to be a type-2 diabetes marker.	Chandrasekaran et al. (2020)

AAS Amino acids score, *ALB-RF* Albumin-rich fraction from pea, *IT* Inlet air temperature, *D50* mean particle size (50% of total particles), *FW* Fresh weight, *GAE* Gallic acid equivalents, *GLB-RF* globulin-rich fraction from pea, *HMW* high molecular weight proteins (>85 kDa), *LMW* Low molecular weight proteins (<20 kDa), *MMW* Medium molecular weight proteins (>20 kDa but <85 kDa), *OAT* Outlet air temperature, *WPI* whey protein isolate

0.52-0.57 g of protein per kilogram of weight (WHO 1979). It has been observed that adequate nutrition helps to reduce inflammation, oxidative stress, and particularly a correct protein supplementation allows the adequate formation of antibodies necessary for defense against microorganisms (Iddir et al. 2020).

Proteins of plant origin can be an alternative to the consumption of animal protein for many people today, especially for the growing sector of vegetarians in the world (Hadnadjev et al. 2017). Plant protein production may have a significant impact on the environment since its emission of CO_2 is lower than with animal protein production (Franzluebbers 2020). There are various sources of protein in oilseeds, cereals, legumes and agri-food waste: it has been observed that the content of essential amino acids in these sources might be lower than that present in animal proteins, particularly amino acids required for muscle formation. However, the essential amino acid profile from soy, oats, corn, potatoes, and brown rice provides the essential amino acids content recommended by the WHO (Day 2016; Gorissen et al. 2018). Also, the named "super foods" as quinoa or chia fulfill the amino acid requirements.

1.5.2 Advantages and Limitations

As stated earlier in this chapter, alkaline extraction–isoelectric precipitation has been the most used method to extract proteins from legumes such as beans, chickpeas, soybeans, broad beans, and lentils. As an advantage, this technique presents high protein recovery since the pH promotes the breaking of the disulfide bridges, also the solubility of the protein increases with an increase in pH. This, in turn, interferes with the folding and stability of the protein. Another advantage is that for laboratory scale, it does not require sophisticated equipment, and a 50-90% of protein recovery is reported using this extraction process (Boye et al. 2010; Kusumah et al. 2020; Gao et al. 2020). As a disadvantage, it is time-consuming, and it is not friendly with the environment since organic solvents, alkali, and acids are used. Extreme alkaline conditions can also affect protein by reducing its digestibility and damaging the amino acids cysteine and lysine and produce toxic substances as lysinoalanine (Schwass and Finley 1984; Hou et al. 2017). Another disadvantage is that pigments such as chlorophyll and polyphenols can also be extracted, resulting in colorful extracts and reduced protein digestibility due to the formation of phlobaphenes and protein–phenolic interactions (Xu and Diosady 2002).

In addition to the conventional methodologies based on water, alkali, detergents, and salts, other separation techniques include reverse micelle, micellar precipitation, aqueous two-phase separation, subcritical water extraction, enzymatic extraction, high pressure, ultrasound (sonication), microwaves, pulsed-electric field, electrostatic separation, among others (Fig. 1.3) (Kumar et al. 2021).

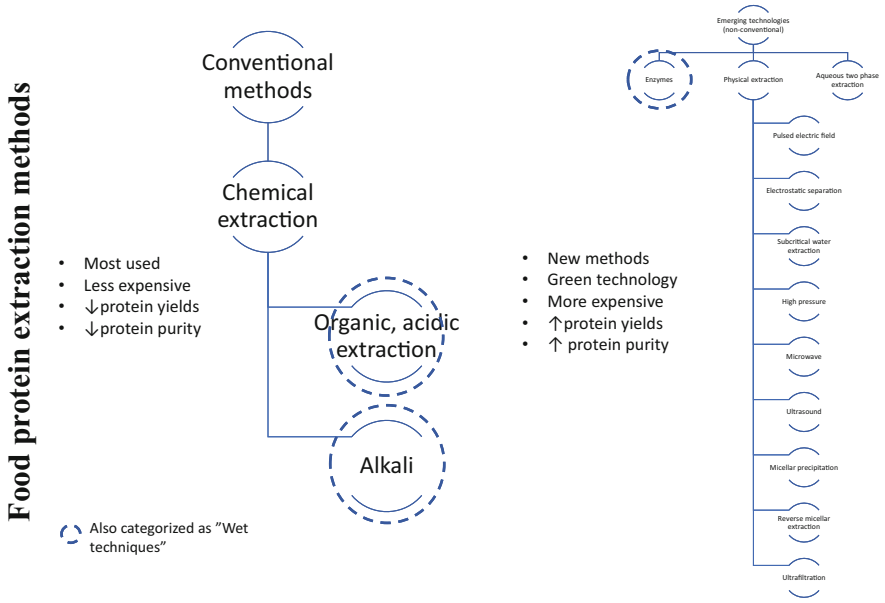

Fig. 1.3 Food protein extraction methods

With these novel methods, also called "unconventional," the recovery and the techno-functional properties of extracted protein are usually better than for the conventional process.

1.5.3 Soy Proteins

One of the legumes that contain the highest amount of protein is soy, with up to 35.9 g per 100 g of dry weight. It is one of the primary sources of food in many countries, and due to its low cost, it is used broadly in food applications (Nazareth et al. 2009). Soy protein isolates and concentrates are among the few commercialized (Ochoa-Rivas et al. 2017). Therefore, the levels of protein recovery with different extraction methods are presented below in Table 1.3.

In Table 1.3, "Green Technologies" are described as lowest use of energy and chemical solvents compared to alkaline extraction (Putnik et al. 2018). The definition of cost is considered at laboratory scale for the equipment, where high pressure, pulsed-electric field, and subcritical water extraction require specialized and expensive equipment, compared with the equipment employed in isoelectric protein precipitation (Fig. 1.2). However, productivity of each process must be taken into consideration.

Many new methods for extracting proteins have shown good recovery levels, at laboratory scale production, but it is critical to evaluate whether the method requires expensive equipment or whether the technology is complicated to upscale at industrial level. Although conventional methods are the most economical, due to their environmental impact, new methodologies for extracting plant proteins must be optimized.

1.6 Conclusions and Perspectives

Alkaline extraction–isoelectric precipitation is a method that yields high protein concentrations from different plant sources. It is based on the solubilization principle of the proteins in alkaline solutions and their isoelectric point. In general, substantial protein denaturation and solubilization occurs at extreme alkaline pH, and the recovered proteins with this methodology have been used for several purposes. However, the main disadvantage of this protocol is the amount of wastewater produced and the low level of protein extraction. Wastewater treatment and reutilization in other processes could diminish the environmental impact of this methodology. Although there are several emerging methods to extract proteins, isoelectric precipitation is the most common method used at industrial level and will still be of interest for the food, feed, and proteins industry due to the increasing demand of protein-based products and a shift to a more plant-based diet. In that sense, the combination of different protein extraction methods can improve the extraction yield, reducing the negative impact of this technology.

Table 1.3 Impact of protein extraction technologies on soybean

Technology	Effect on protein	Yield of recovery	Cost	Green technology	Reference
Isoelectric protein precipitation	Effect on physicochemical characteristics of the protein [MM3]; pH promotes breaking of disulfide bridges and the solubility of the protein, and their solubility	50–90%	Low cost	No	Boye et al. (2010); Kusumah et al. (2020); Gao et al. (2020).
Electrostatic separation	No effect on native conformation of the protein	62%	Low cost	Yes	Xing et al. (2018)
Subcritical water extraction	Improves the protein solubility	59.3%	High cost	Yes	Lu et al. (2016); Pojić et al. (2018)
Aqueous two-phase extraction	High stability and solubility of the proteins	64%	Low cost	Yes	Estela da Silva and Teixeira Franco (2000)
Enzyme-assisted extraction	Effect on physicochemical characteristic of the protein for long processing times, but short processing time reduces the denaturation of the proteins when compared to alkaline extraction.	90%	High cost	Yes	Sari et al. (2013); Lu et al. (2016)
High pressure	Above 200 Mpa, increases hydrophobicity, reduces free sulfhydryl bonds, and changes the secondary structure.	82%	High cost	Yes	Puppo et al. (2004); Preece et al. (2017)
Pulsed-electric field	Induces dissociation, denaturation, and reaggregation, but no impact on the secondary structure	ND	High cost	Yes	Li et al. (2007); Sampedro et al. (2014)
Microwave-assisted extraction	Changes in the secondary structure of the protein in β-sheet have been observed	58%	Low cost	Yes	Choi et al. (2006); Ochoa-Rivas et al. (2017)
Micellar precipitation [a]	Reduction of surface hydrophobicity and protein solubility.	81.9–87.8%	Low cost	Yes	Stone et al. (2015)
Reverse micelle extraction	The internal aqueous nuclei of the reverse micelles solubilize	72.4%	Low cost	Yes	Zhao et al. (2015)

(continued)

Table 1.3 (continued)

Technology	Effect on protein	Yield of recovery	Cost	Green technology	Reference
	hydrophilic proteins. Soy contains several globulins that have different characteristics of hydrophobicity and disulfide bridges, which is related to its aggregation properties, so the extraction process becomes essential so that the protein preserves or acquires new physicochemical properties for its subsequent consumption				
Ultrasound-assisted extraction	Changes in protein structure, denaturation, formation of new covalent bounds	57.27–72.91%	Low cost	Yes	Tiwari (2015); Man et al. (2017)
Ultrafiltration	Depending on the membrane used to ultrafiltrate, electrostatic properties of the proteins can be modified	70–92.2%	Low cost	Yes	Ali et al. (2010); Baldasso et al. (2011)

ND No Data, *Mpa* megapascal
[a]This study was conducted using whey proteins since no literature was available for soybean proteins

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Air Classification of Plant Proteins

2

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Abstract

Dry milling followed by air classification has been widely adapted as a sustainable and energy-friendly approach for enrichment of plant proteins from various agro-materials including legumes, oilseed meals, cereals, and brans. Dry milling is a critical stage as it should optimally reduce the size of protein particles to fine levels while disentangling them from the remaining coarse starch- and/or fiber-enriched particles. Fractionation of the fine protein-rich particles from the coarse carbohydrate-rich particles can be performed as a function of their size and density through air classification. Centrifugal air classifiers are among the most commonly used modern classifiers for partial separation of plant proteins from various agro-materials. Protein enrichment level and its separation efficiency are affected by the milling types and intensities as well as air classifier wheel speeds. While moderate to intense milling speeds of ~4000 rpm were found optimal in maximizing protein enrichment of starch-rich legumes, mild milling speeds of ~1000 rpm or less were essential for optimal protein enrichment of non-starch legumes and oilseed meals. Air classification utilizes a water- and chemical-free environment with no high temperature stress and pH shifts and can produce a

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variety of fractions with unique functional properties with applications in novel food production systems such as 3D food printing and space foods. Air classified protein concentrates fractionated at their native states exhibited low viscosity, but improved solubility, emulsifying and foaming properties compared to wet-fractionated protein isolates/concentrates. Air classified protein-depleted fractions also exhibited relatively high water holding capacity (WHC) and gelatinization behavior.

Keywords

Air classification · Dry milling · Milling intensity · Plant protein · Starch granules · Functional properties · Protein (fine) fraction · Carbohydrate (coarse) fraction

2.1 Introduction to Air Classification, Scope, and Approach

Plant-derived constituents such as protein, starch, and fiber are conventionally obtained through wet fractionation processes that use harsh acidic or alkaline reagents with extreme pH shifts while requiring substantial energy for freeze/spray-drying (Assatory et al. 2019; Schutyser and van der Goot 2011). Wet fractionation techniques can produce protein isolates with high purities above 90%; however, their extraction conditions lead to the alteration of a protein's native structure, functionality, and solubility along with the detrimental consequences on the environment (Jafari et al. 2016; Tabtabaei et al. 2019).

Traditional wet extraction processes consist of solubilization of the agro-material flours at alkaline pH followed by acid precipitation of the solubilized plant proteins before freeze/spray-drying. The application of this technology to oilseeds requires their oil removal before protein extraction which is usually performed using hexane, ethyl alcohol, isopropyl alcohol, or super critical carbon dioxide. The resulting oilseed meals should go through an additional thermal desolventization process before protein extraction which is known to significantly denature the extracted proteins (Balke 2006; Soltero 2013; Tabtabaei 2015). Aqueous or enzyme-assisted aqueous extraction processes (AEP/EAEP) are "green" technologies developed to eliminate solvent use and defatting (Campbell et al. 2011; de Moura et al. 2011, 2009, 2008; de Moura and Johnson 2009; Jung et al. 2009; Rosenthal et al. 1998; Tabtabaei and Diosady 2013). During AEP/EAEP processes, water is being used to solubilize protein and other soluble components in oilseed flours/grits while releasing oil as a separate phase through centrifugation processes. The resulting protein-rich skim fractions can be further concentrated and purified through additional ultrafiltration and diafiltration membrane processes to produce high-quality protein isolates and concentrates with improved functionalities and no solvent residues (Tabtabaei et al. 2017a). While AEP/EAEP processes eliminate solvent utilization, they still require the use of water along with acid and base solutions as well as energy-intensive dehydration steps. They also introduce new challenges to the industry due to the formation of very stable oil-in-water emulsions stabilized by

natural emulsifiers of oleosin proteins, storage proteins, and phospholipids which complicates their destabilization to free oil (Chabrand et al. 2008; Chabrand and Glatz 2009; Tabtabaei et al. 2015, 2014, 2013; Tabtabaei and Diosady 2012).

Alternatively, energy-friendly dry fractionation techniques are evaluated as chemical-free processes compared to the wet fractionation methods. Air classification and electrostatic separations are among the well-studied approaches of dry fractionation for plant components. In comparison with the wet extraction, dry fractionation requires no water and consumes less energy, while preserving the native state and functional properties of the constituent fractions. Dry fractionation is not capable of producing isolates of high purity; however, most of the food manufacturing processes typically need a reasonable concentration of protein/starch-rich additives in the development of desirable product structure (Tabtabaei et al. 2016a).

Air has long been used as a carrier gas to separate the particles, and with the invention of air classifiers, its application has broadened to fractionate heterogeneous particulates based on their size and density (Vose 1978). Air classification has been effectively used to concentrate plant proteins from pea beans, northern beans, faba beans, field peas, mung beans, lima beans, and lentils (Sosulski and Youngs 1979). The plant proteins derived by air classification have superior functional properties and are suitable for production of a variety of solid, semi-solid, and liquid food products as emulsifiers and/or foaming agents (Assatory et al. 2019; Pelgrom et al. 2014).

The primary purpose of this chapter is to explore the application of air classification in plant protein production. The chapter will include the theory and mechanism of air classification, the parameters affecting the efficiency of the process, and the functionality of air classified plant proteins along with the advantages and limitations of this technique.

2.2 Air Classification Theory and Principles

The most prevalent type of dry fractionation technology is the combination of dry milling and air classification. Dry milling along with air classification processes has been long used to extract fractions with various particle sizes and nutritional contents from a variety of agro-materials such as legumes, oilseed meals, cereal bran, and endosperm flours (Andersson et al. 2000). First, small protein particles are physically disentangled from large starch granules or other non-starch polysaccharides and fibers through dry milling. Next, the protein-rich fraction (light fine fraction) is separated from the starch- or fiber-rich fractions (heavy coarse fraction) in a circulating air stream based on the size and density of the particles (Boye et al. 2010). The separation mechanism in air classification is based on the aerodynamic properties of particles (size and density), and it works by introducing air currents into a chamber that is holding milled agro-materials. The air promotes centrifugal and/or gravitational forces that lead to the separation of the different particles as a result of their contrasting physical and chemical characteristics (Day 2013; Pelgrom et al.

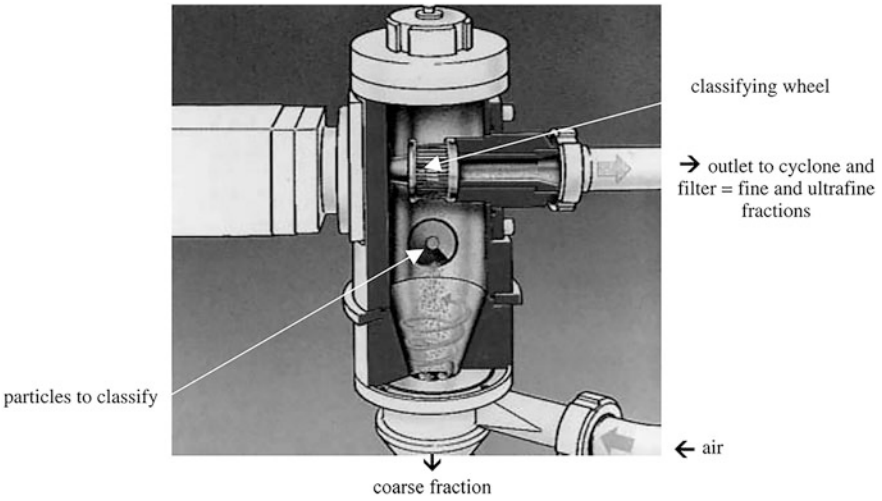

Fig. 2.1 Schematic representation of air classification, Hosokawa-Alpine 50 ATP turboplex classifier (Létang et al. 2002)

2013). The sample is fractionated into coarse and fine fractions where the former represents the particles that were not moved by air while the latter group experiences translocation. Through air classification, milled agro-materials can be split into several fractions by adjusting the air speed and pressure. The effective separation of protein-, starch-, and fiber-enriched fractions from agro-materials through dry-based fractionation techniques, such as air classification and electrostatic separation, relies heavily on the milling process. Proper milling not only could reduce the particle size and expose cellular components but also could promote successful disentanglement of protein, starch, and other plant cell contents. A brief review of various milling types and the factors affecting the milling quality will be discussed later in this book chapter.

Different types of air classifiers have been developed to separate various particles in the food, pharmaceutical, cement, coal, plastic, fertilizer, and wood industries. The most popularly used air classifiers are gravitational, cascade, inertial, fluidized bed, and centrifugal air classifiers. Gravitational air classifiers are among the early countercurrent and crossflow classifiers operating with rising air flow and horizontal air flow, respectively. Centrifugal air classifiers are modern classifiers operating with vortex chamber, orientable blades, rotating blades, or fans (Shapiro and Galperin 2005). Gravitational-centrifugal air classifiers are widely used for separation of plant protein particles from various agro-materials (Létang et al. 2002; Pelgrom et al. 2015b; 2014; 2013).

The schematic visualization of the most common gravitational-centrifugal air classifier (Hosokawa-Alpine 50 ATP turboplex) is presented in Fig. 2.1. Milled particles are introduced from the top of the chamber and transported by airflow to the rotating classifier wheel with slits. While the wheel axis of traditional air classifiers

runs vertically, the one in ATP model moves horizontally. Fine and light protein-rich particles move to the top of the air classifier due to the prevailing drag force generated by the air (Shapiro and Galperin 2005). The particles below the cut-off point pass through the slits and are taken to the cyclone where fine and ultrafine fractions are captured by a filter (Létang et al. 2002). The coarse and heavy particles (i.e. starch granules) move to the bottom of the unit due to the prevailing gravity force caused by rotational speed; thus large particles are collected in a tank beneath the chamber when they leave the unit (Létang et al. 2002; Shapiro and Galperin 2005).

The cut-off point in air classification is described as a particle diameter on the Tromp curve, T (x) in Eq. 2.1, that has a 50/50 possibility of falling into a fine or coarse fraction (Pelgrom et al. 2013), which happens when the centrifugal and drag forces are equal to each other (Bauder et al. 2004). The cut-off point can be technically changed by managing the speed of the classifying wheel and the airflow. Constructing a Tromp curve through Eqs. (2.1) and (2.2) can help to calculate the cut-off point (Leschonski 1984; Pelgrom et al. 2013). The Tromp curve represents a portion of the feed passed to underflow (coarse) or overflow (fine) streams at a given particle size (Altun and Benzer 2014).

$$T(x) = \frac{g \times q_G(x)}{q_A(x)} \tag{2.1}$$

$$g = \frac{weight(coarse\ fraction)}{weight(coarse\ fraction) + weight(fine\ fraction)} \tag{2.2}$$

where x is the size of a particle, $T(x)$ is the probability of the feed passing to either coarse or fine stream, $q_G(x)$ is the frequency particle size distribution of the coarse fraction, $q_A(x)$ is the frequency particle size distribution of the feed, and g (Eq. 2.2) is the weight ratio of coarse fraction to both coarse and fine fractions collected after air classification. Thus, knowing the particle size distributions and yields for the fine, coarse, and feed materials can help to plot the Tromp curve and then experimentally calculate the cut-off point of the air classification process that corresponds to the particle size (x) on the Tromp curve having T(x) value of 0.5 or 50%.

2.3 Parameters Affecting Air Classification

Several factors affect the separation efficiency of the air classification process as presented in the following sub-sections. Separation efficiency can be calculated as the percentage of the subject component present in the fraction heavily enriched in that component divided by that component content initially present in the starting flour. Protein separation efficiency (Eq. 2.3) is obtained by dividing the total protein in an air classified protein-rich (fine) fraction by the protein amount in the original feed.

$$\text{Protein separation efficiency } (\%) = (\text{protein (g) in air classified fine fraction}/\text{protein (g) in the feed}) \times 100 \quad (2.3)$$

2.3.1 Milling Types and Factors Affecting Milling Quality

Successful fractionations of agro-materials through air classification require the constituents of the plant cell to be sufficiently small and disaggregated through the milling process (Andersson et al. 2000). The effectiveness of particle size reduction and the exposure of the cell contents depend on the milling technology and their operating conditions. Several technologies, such as impact milling (hammer and pin mills), attrition milling, and jet milling have been commonly used for crushing and pulverizing grains, cereals, and legumes. In impact milling, for example, a hammer mill pulverizes the legumes and grains using rapidly rotating hammers until the particles are small enough to pass through an adjacent screen. The working principles of pin milling are similar to hammer milling, except that particles are impacted against an intermeshing pin disk rotating at a faster speed. In attrition milling, a cage-like rotor is used not only to impact but also to shear and cut the particles intensively. Research has shown that the flours crushed by attrition milling have less starch damage compared to pin-milled flours (Sosulski et al. 1988). More recently, jet mills have been developed to produce ultrafine particles using high-velocity gas and by colliding the particles against one another and with the wall without a need for moving or rotating discs or hammers (Chen et al. 2013; Létang et al. 2002; Sadler et al. 1975; Schell and Harwood 1994). Létang et al. (2002) explored the effect of milling on the air classification efficiency, and the results showed that jet-milled feed could enhance starch-protein separation of hard and soft wheat flours and lower energy dissipation compared to those obtained by pin milling since the grinding occurs mainly by the inter-particle collisions. They observed that the protein content of starch-rich fractions could be reduced to less than 2% with a series of grinding and air classifications, i.e., 3 and 5 grinding steps for the soft and hard wheat flour, respectively.

The effective disentanglement of protein particles from starch granules through milling could also be influenced by several factors, such as milling speed and intensity as well as agro-material's type, composition, and seed hardness (Schutyser and van der Goot 2011). The effect of milling parameters on the separation of starch granules from the cell wall and protein bodies for starch-rich legumes such as pea, bean, lentil, and chickpea was examined by Pelgrom et al. (2015a). In this study, the milling parameters were optimized for a better disentanglement of starch granules and separation from other cellular components using air classification. The efficiency of dry-based protein fraction is also affected by the size of starch granules compared to the other constituent fractions (Cloutt et al. 1987; Tyler 1984). For example, starch granules of legumes cotyledon tissues as well as wheat and barley endosperms are normally larger (20–35 μm in diameter) than protein bodies (less than 20 μm in

diameter), which allows for an effective separation during the dry fractionation process (Delcour and Hoseney 2010; Schutyser and van der Goot 2011). The efficiency of dry fractionation processes decreases once the size of starch granules is small (for example, 3–8 μm in diameter in rice and chickpea) and is close to the cut-point for separating the protein-rich particles (Delcour and Hoseney 2010; Pelgrom et al. 2015a).

Milling speed controls the level of particles disaggregation, which results in effective separation during dry fractionation. For instance, increased speed of milling has shown to improve the protein content of fine fractions in yellow pea, allowing only particles with a similar size to protein bodies to pass the classifier (Pelgrom et al. 2013). In this case, increasing the classifier speed from 2500 rpm to 8000 rpm raised the protein content of the yellow pea fine fractions from 22.5% to 32.7% in jet milling and from 22.4% to 55.4% in the impact milling. The possible reason may be that a sufficient detachment of proteins from the starch granules occurs at a higher milling speed. Nevertheless, over-milled feed flour can be too fine in size and have an excessive surface area that might cause the severe attraction between particles due to van der Waals forces and a lack of cohesion in a flow (Dijkink et al. 2007; Pelgrom et al. 2014; 2013). Furthermore, such heavily milled powder can adhere to the mill wall, thus reducing the overall milling yield. Over-milling was also found to damage starch granules which eventually lowers the air classification separation efficiency by moving damaged starch granules to protein-enriched (fine) fractions (Pelgrom et al. 2014).

Milling intensity is described by the number of passes the feed proceeds through the grinding machine. A study on field peas by Wu and Nichols (2005) showed that increased intensity of milling before air classification resulted in a higher yield of protein-rich fraction and higher starch purity of starch-rich fraction. In another study, the highest content of β-glucan (dietary fiber) was achieved at 3 × 14,000 rpm rather than at 1 × 14,000 rpm or 1 × 9000 rpm (Wu and Doehlert 2002).

Other factors that affect the efficiency of milling for the disentanglement of protein, starch, and other plant cell contents, and, therefore, the efficiency of the air classification are the chemical composition and hardness of the seeds. For example, the presence of high water-insoluble cell wall components and crude fiber in legume cotyledons could result in less effective milling of the cell contents, which makes the flour more resistant to size reduction by pin milling (Tyler 1984). Tyler (1984) also reported that the hardness of legume seeds was inversely associated with the efficient disentanglement of protein bodies from the starch granules, which eventually lowered the separation efficiency during the air classification process. In another study, Létang et al. (2002) demonstrated that hard wheat was more challenging to mill than soft wheat. A stronger bind of starch and protein molecules in hard wheat particles makes the grinding and separation more difficult than soft wheat (Delcour and Hoseney 2010). Létang et al. (2002) observed lower milling yields and more starch damage during the milling of hard wheat and eventually poorer protein enrichment during the classification process. Similarly, Pelgrom et al. (2015a) observed that bean (*Phaseolus vulgaris*) and lentil (*Lens culinaris*) produce smaller particle diameters compared to pea (*Pisum sativum*) and

chickpea (*Cicer arietinum*) after the milling process, as bean and lentil had lower hardness than pea and chickpeas. As a result they obtained the highest protein content in the fine fractions of the seeds with lower hardness, such as lentils. In general, seeds with higher hardness factors hinder milling and lower the efficiency of the milling process. Therefore, the efficiency of the milling process to disentangle the starch granules from protein bodies and other constituent fractions depends on how strong starch and protein molecules bind, suggesting that hardness could control not only the size of protein bodies and starch granules but also the compositions of particles and their distribution after milling.

2.3.2 Classifier Speed

Classifier wheel or rotor speed has been found as one of the important operating conditions during air classification of agro-materials. As classifier wheel speed increases, fewer small particles can pass through the wheel to make the fine fractions, thus ending up in the coarse fraction. This can generally reduce the mean particle size diameters of the collected fine and coarse fractions while improving their protein enrichment. However, the protein separation efficiency of the fine fraction may decline due to reduced mass yield.

The influence of air classifier wheel speed on pea protein enrichment was studied (Pelgrom et al. 2013) by increasing the wheel speeds from 5000 to 12,000 rpm that resulted in reducing the mean particle size diameters (D_{50}) of the fine fractions (from 8.8 to 4.8 μm) and coarse fractions (from 23.7 to 19.2 μm). The protein content of the fine and coarse fractions was slightly improved. For the fine fractions, the protein content increased from ~50% to 55%, and for the coarse fractions, the increased level of protein enrichment was from ~10% to 20%. However, the protein separation efficiencies of the fine fractions were significantly declined at higher classifer speeds (from 76.8% to 29.2%) due to reduced mass yields.

In another study, increased speed of the air classifier wheel from 7000 to 13,000 rpm during air classification of milled lupine flour (low-intensity impact milling at 1000 rpm) has shown to decrease the particle size of the fine fractions from 11.4 to 6.3 μm, while increasing their protein content from 53.7% to 58.9% (Pelgrom et al. 2014). However, as with a milling speed, a higher rate of classifier speed resulted in a lack of cohesive flow and fouling of classifier walls which eventually lowered the overall yield of separation (Pelgrom et al. 2014, 2013).

Through another study performed by Sibakov et al. (2011) on the pilot scale, the non-defatted and defatted (using supercritical carbon dioxide, SC-CO2) oat flakes/grits were initially milled and air classified at different classifier wheel speeds to produce coarse bran-rich and fine endosperm-rich oat fractions. The resulting bran-rich oat fractions went through additional milling and air classification processes at different classifier wheel speeds with the aim of enriching the coarse fractions in β-glucan. For defatted oat flakes, the first-stage air classification was performed at various classifier wheel speeds of 3000 to 7000 rpm where the yields of the air classified coarse fractions increased significantly from 9% to 24.4% while their

β-glucan concentrations reduced from 23.3 to 13%. Air classification at higher speeds has also reduced the average particle size (D_{50}) of the coarse fractions from 435 to 236 μm. The coarse fraction obtained at 4000 rpm with 15% β-glucan content was introduced to the second stage milling and air classification processes at different classifier speeds of 2850 to 4000 rpm that resulted in the production of coarse fractions with yields increasing from 5.2% to 10.4%, but almost similar β-glucan contents (30–31.2%) and particle sizes (D_{50}: 254–300 μm). For non-defatted oat flakes, the air classification at different wheel speeds of 1800 to 2500 rpm resulted in low particle size coarse fractions with improved mass yields, but they all obtained similar β-glucan concentrations regardless of the classifier wheel speeds. This different separation behavior of defatted and non-defatted oat grits can be attributed to their initial oil content that was also found as an important factor affecting the efficiency of air classification process as described in Sects. 2.3.3 and 2.3.5.

2.3.3 Chemical and Physical Properties of Starting Agro-Material

Chemical composition and physical properties of the starting agro-materials are among parameters that could affect air classification. Xing et al. (2020) have reported that the oil content and starch granule size distribution of the feed material could highly affect protein separation efficiency during air classification. Their study showed that the air classification of chickpea was less efficient compared to pea and lentil. This was attributed not only to a higher amount of oil in chickpea (6%, dry basis) that resulted in a higher agglomeration level compared to peas and lentils (1%, dry basis), but also to the smaller starch granules of chickpea (~22 μm) compared to pea and lentil (25 and 23 μm, respectively). Starch granules can end up in the fine fractions if their size is close to the cut-off point in the air classification, thus reducing protein enrichment level in the fine fractions. Additionally, particles with higher lipid content are harder to be separated by air classification. Other studies also found that high lipid content can cause agglomeration of milled particles and prevent free dispersion in the air classifier (Dijkink et al. 2007; Schutyser and van der Goot 2011).

Three distinctive layers (palisade, hourglass, and parenchyma cells) of soybean hulls were affected differently in each collected sample after sieving, pin milling, and air classification (Wolf et al. 2002). The palisade cells were found to be difficult to break down and to be resistant to pin milling. These cellular structures can be a challenge for air classification, hence specific structures such as hulls are usually removed (Pelgrom et al. 2015c). Wu and Nichols (2005), as part of their successive pin milling and air classification (9 × 14,000 rpm) approach, found that dehulled field pea results in higher fine fraction yield of 34.3% with slightly more protein concentration of 52.7% compared to the fine fraction yield of 28.9% and protein content of 52% for whole pea.

2.3.4 Moisture Content

The moisture content of the feed also affects the separation efficiency of air classification, by affecting the particle sizes during grinding (Dijkink and Langelaan 2002; Schorno et al. 2009). Lower moisture content has shown to increase the yield of fine fraction for lupine and pea flours but at the cost of protein content in protein-enriched fraction (Pelgrom et al. 2015c, a). High yield was associated with the brittleness of low-moisture particles, which allowed milling to produce more fine particles.

2.3.5 Pre-Treatments Prior to Air Classification

There are various pre-treatments that could promote the separation efficiency of air classification. Pelgrom et al. (2015c) applied several pre-treatments, such as defatting, prior to the air classification of lupine and pea grits. The oil-rich lupine grits with 35.1% protein and 7.3% oil were defatted with petroleum ether where about half of their oil was separated before entering the air classifier. The defatting process substantially increased the protein purity of the fine fraction from ~45% (dry basis, non-defatted) to ~57% (dry basis, defatted). The yield and protein content of the fine fractions obtained after air classification of non-defatted and defatted pea grits were almost similar due to low oil concentration in both non-defatted (1.7%) and defatted (0.7%) pea. As it was described earlier, reduced lipid content promoted greater dispersion of particles in the air which resulted in easier starch-protein separation (Dijkink et al. 2007; Schutyser and van der Goot 2011). Methods such as supercritical carbon dioxide extraction have been also applied for the removal of oil from oat flakes before milling and air classification that enabled the more efficient separation of β-glucan in the cell wall-enriched coarse fraction. It was believed that oil removal prior to air classification could facilitate liberation of starch granules from the cellular structure, resulting in production of a more enriched β-glucan coarse fraction (Sibakov et al. 2011).

Reducing moisture content by drying was another pre-treatment step applied to lupine and pea flours prior to the classification, which increased the yield of the fine fraction but lowered its protein content (Pelgrom et al. 2015c). Pre-soaking the feed flours decreased the particle density due to the protein dissolution while soaked (Pelgrom et al. 2015c). Such decreased particle density made the separation harder as it resulted in lower protein content of the target fraction. Freezing the soaked feed further decreased its density, increased the size of particles, and eventually lowered the protein purity of the protein-rich fraction.

Pelgrom et al. (2014) have also applied flowability aids (40% potato starch or 1% Aerosil) in the air classifier to enhance the dispersibility of lupine flour in the air, thus increasing the separation efficiency of lupine protein. Aerosil nanoparticles did improve the dispersibility of lupine flour by coating the surface of the flour particles, resulted in improving the protein separation efficiency of the fine fraction by ~11% but sacrificing its protein enrichment level by ~10%. While the use of starch microparticles as carriers for lupine flour smaller particles did improve their

flowability in the air classifier, they did negatively affect the yield and protein purity of the resulting fine fraction after air classification. The application of such aids lowers attractive forces and increases the distance between particles (Müller et al. 2008).

2.4 Applications in Plant Protein Separation

Over the past few decades, several applications of air classification to various processes have been realized with extensive implementation in commercial settings, namely, air pollution control, food processing, pharmaceutical, cosmetic and chemical industries. Within the context of food research, studies have been conducted on corn fiber, soybean hulls, several different types of peas, pinto beans, wheat, barley, and oat bran with varying results and efficiencies attributed to the diversity in the aforementioned characteristics. Table 2.1 provides a summary of air classification application in different studies of plant protein, starch, and fiber enrichment. Possible applications of collected protein-rich fractions are the food industry, while starch-rich fractions could be utilized for the fermentation of ethanol (Wu and Nichols 2005). For example, extrusion of air classified starch-rich fraction of pinto bean made the flour high in dietary fiber and lysine, and low in total fat; this can improve the nutritional profile of cereals and other snacks (Simons et al. 2017).

As can be seen from Table 2.1, the air classification has been tested so far on different agro-materials including oilseed hulls (Wolf et al. 2002), oilseed meals (Challa et al. 2010; Laudadio et al. 2013), starch-rich legumes (Coda et al. 2015; Diedericks et al. 2020; Pelgrom et al. 2015b, c; a; Simons et al. 2017; Wu and Nichols 2005; Xing et al. 2020; Zhu et al. 2020), non-starch legumes (Pelgrom et al. 2015c; 2014), cereal brans (Silventoinen et al. 2021, 2019; Wu and Doehlert 2002), and cereal endosperms (Andersson et al. 2000; Létang et al. 2002; Sibakov et al. 2011; Silventoinen et al. 2018; Wu et al. 1994).

Air classification of oilseed hulls such as soybean hulls increased the starting protein content from 15% to 43% but with a yield of only 2% (Wolf et al. 2002), while the protein-rich fraction of sunflower meal from oilseed meals accounted for 88% yield and its protein content increased from 34% to 40% (Laudadio et al. 2013). From Table 2.1, it can be seen that most of the research has been done on starch-rich legumes where yellow pea had an initial protein content of 22% which was increased to 57% accounting for 25% of the flour weight (Xing et al. 2020). Eclipse pea (starch-rich legume) almost displayed the same increase in protein content from 23% to 56% but with a lower yield of 13% (Wu and Nichols 2005). Air classification of non-starch legumes such as lupine (protein content 35%) resulted in a protein-rich fraction with 57% protein and 21% yield (Pelgrom et al. 2015c). One study on rye bran as a model cereal bran showed an increase of protein content from 15% to 31%, accounting for 13% yield. Air classification of cereal endosperms such as Prowashonupana barley with a 22% starting protein content resulted in 48% protein enrichment and a relatively low yield of 6% (Wu et al. 1994).

Table 2.1 Applications of air classification for various plant materials

Feed	Milling	Initial Content (% dry basis)	Enriched fraction			Reference
			Highest Content (% dry basis)	Particle Size (μm)	Yield (%)	
Corn fiber	Pin mill	Protein: 13.1	17.1	<15	7.9	Wu and Norton (2001)
		Starch: 15.4	30.2	15–18	10.4	
Soybean hulls	Pin mill	Protein: 14.6	43.3	<15	2.0	Wolf et al. (2002)
Oat bran defatted	Pin mill	Fiber [a]: 6.1	18.2	>30	39.3	Wu and Doehlert (2002)
Whole Eclipse pea	Pin mill	Protein: 22.9	55.9	<15	12.6	Wu and Nichols (2005)
		Starch: 46.2	73.7	24–30	21.6	
Oat grain dehulled and defatted	Pin mill	Fiber [a]: 3.2 ± 0.3	33.9 ± 0.2	197	7.8	Sibakov et al. (2011)
Yellow pea	Pin and impact mills	Protein: 23.0	42.9 ± 0.2	–	–	Pelgrom et al. (2015b)
		Starch: 44.0	67.2 ± 1.6			
Yellow pea	Impact mill	Protein: 21.9	43.9 ± 1.2	9.9 ± 0.5	32.5 ± 0.4	Pelgrom et al. (2015c)
Lupine defatted		Protein: 35.1 ± 1.9	56.9 ± 2.0	12.4 ± 1.2	21.3 ± 2.3	
Pea (*Pisum sativum*)	Impact mill	Protein: 23.7 ± 0.8	55.6 ± 0.5	5.4	–	Pelgrom et al. (2015a)
Bean (*Phaseolus vulgaris*)		Protein: 29.8 ± 1.4	52.8 ± 0.3	5.1 ± 0.1		
Chickpea		Protein: 21.6 ± 0.9	45.3 ± 0.7	7.8 ± 0.4		
Lentil		Protein: 24.9 ± 0.3	58.5 ± 0.2	5.3 ± 0.2		
Pinto bean (non-extruded)	Pin mill	Starch: 46.4 ± 3.1	56.0 ± 4.1	15–45	–	Simons et al. (2017)
Barley endosperm	No mill	Protein: 8.3 ± 0.1	28.3 ± 0.3	3.2	6.4 ± 0.7	Silventoinen et al. (2018)
		Starch: 80.0	90.3 ± 0.1	22.0 ± 0.1	69.0	

Yellow pea	Pin and impact mills	Protein: 21.9 ± 1.5	57.1 ± 0.2	–	24.5 ± 1.1	Xing et al. (2020)
Sunflower meal	Micronization	Protein: 34.0	40.0	–	87.9	Laudadio et al. (2013)
Wheat bran	Pin mill	Protein: 16.4 ± 0.3	30.9 ± 0.7	–	9.6 ± 0.7	Silventoinen et al. (2021)
Rye bran		Protein: 14.7 ± 0.1	30.7 ± 0.5		12.9 ± 0.3	
Bz 489–30 naked barley	Pin mill	Protein: 13.5	20.6	–	29.6	Andersson et al. (2000)
Prowashonupana hulled barley defatted	Pin mill	Protein: 22.4 ± 0.1	47.8	–	6	Wu et al. (1994)
		Fiber [a]: 19.6 ± 0.6	37.9 ± 0.2	24–30	5	
Soft wheat flour (commercial)	Jet mill	Protein: 11.4	20.7	6.5	34.4	Létang et al. (2002)
Hard wheat flour (commercial)		Protein: 12.5	22.9	5.9	31.2	
Soybean meal	No mill (sieving)	Protein: 53.9	55.7	–	91.0	Challa et al. (2010)
		Fiber [b]: 8.1	50.2		4.0	
Cottonseed meal		Protein: 49.9	52.7		99.0	
		Fiber [b]: 22.6	38.5		1.0	
Wheat middlings		Protein: 19.2	20.1		95.0	
		Fiber [b]: 39.6	58.1		5.0	
Rice bran	Impact and pin mills	Protein: 18.5 ± 0.5	27.4 ± 0.2	5.6 ± 0.2	13.9 ± 0.1	Silventoinen et al. (2019)
Faba bean	Impact mill	Protein: 35.7 ± 0.4	51.5 ± 0.25	11.9 ± 0.1	46.7	Coda et al. (2015)
		Starch: 42.2 ± 0.8	65.8 ± 0.5	23.8 ± 0.2	52.4	
Bambara groundnut dehulled	Rotor mill	Protein: 17.7	32.6 ± 0.2	17.9	16.2	Diedericks et al. (2020)
Lupine	Pin and impact mills	Protein: 38.0	58.9	6.3	6.1 ± 0.1	Pelgrom et al. (2014)

(continued)

Table 2.1 (continued)

Feed	Milling	Initial Content (% dry basis)	Enriched fraction			Reference
			Highest Content (% dry basis)	Particle Size (μm)	Yield (%)	
Mung bean	Impact mill	Protein: 23.4 ± 0.9	57.6 ± 0.9	–	16.2 ± 0.9	Zhu et al. (2020)
Soybean meal	Pin mill	Protein: 52.5	54.2	–	72	Sosulski and Youngs (1979)
Lupine		Protein: 41.4	43.3		82	
Chickpea		Protein: 19.5	28.9		29	
Pea beans		Protein: 24.7	52.4		26	
Northern pea		Protein: 24.0	53.5		22.5	
Faba bean		Protein: 29.8	66.6		28	
Field pea		Protein: 25.3	61.3		24	
Lima bean		Protein: 23.0	47.7		27	
Mung bean		Protein: 26.5	60.4		29	
Lentil		Protein: 23.9	57.9		26	

[a] β-glucan
[b] neutral detergent fiber

From air classification results (Table 2.1), it seems that the air classification of starch-rich legumes resulted in higher protein enrichment in the resulting protein-rich (fine) fractions. Among starch-rich legumes (Table 2.1), the maximum protein purity was achieved in yellow pea with 2.6 times enrichment (Xing et al. 2020) and the minimum protein purity was observed in chickpea with less than 1.5 times enrichment (Sosulski and Youngs 1979).

The high enrichment of proteins in starch-rich legumes might be attributed to their cotyledon structures that mainly consisted of less brittle starch granules (20–40 μm in diameter) embedded in a friable matrix of proteins (Aguilera et al. 1984; Aguilera et al. 1982; Pelgrom et al. 2015a; Tyler et al. 1981). The presence of resilient starch granules with relatively large diameters enables optimal dry milling not only to reduce size of the protein matrix, but also to remove most of the protein matrix from the surface of starch granules while leaving the starch granules intact. Therefore, optimal milling of legumes can result in production of a starting flour with broader size distribution through which milled particles can be easily separated by air classification based on differences in their size and density.

Obtaining milled flours with broader size particle distributions through dry milling is generally difficult for non-starch legumes and oilseed meals as they are mainly composed of protein bodies and other non-starch polysaccharides and fibers. Therefore, their milling especially at intense settings can result in production of milled particles with narrow size distributions and limited variation in size and/or density, decreasing the protein enrichment during air classification. Pelgrom et al. (2014) explored the effect of milling on air classification of lupine flours and proposed the very coarse milling at low classifier wheel speeds of ~1000 rpm to produce starting flour with broader size distribution that its protein content could be increased from ~38% to ~59% after air classification. Other studies involving defatted oilseeds of soybean, sunflower, and cotton seed meals did result in very minimal protein enrichment and obtaining very high yields of the fine fractions (Challa et al. 2010; Laudadio et al. 2013; Sosulski and Youngs 1979). This is attributed to the accumulation of both protein particles and non-protein particles in the fine fractions after air classification since the milling of those agro-materials did fail to only reduce the size of the protein particles while leaving rest of the fiber and non-starch polysaccharides as larger particle groups.

2.5 Food Functionality of Air Classified Protein-Rich Fractions

Food functionality is a non-nutritional property of protein products and ingredients affecting their utilization and application in different food production systems (Han and Khan 1990). Air classified protein-enriched (fine) fractions can preserve their native bio-functionality during dry milling and air classification and have been shown to possess superior nitrogen solubility index (NSI), emulsification, gelation, and foaming properties not only compared to starting milled flour and air classified starch-rich (coarse) fractions, but also compared to those protein isolates and concentrates obtained by wet fractionation techniques (Bergthaller et al. 2001; Han

and Khan 1990; Pelgrom et al. 2015b, 2014, 2013; Sosulski and McCurdy 1987; Sosulski and Youngs 1979). The superior and native functionality of air classified protein-enriched fractions can potentially promote the production of novel and healthier food products with desirable structural and physical properties through green and energy-friendly sources and processes.

Food functional properties are generally investigated in terms of nitrogen solubility index (NSI), water holding capacity (WHC), emulsification, whip-ability, water and oil absorption capacities (WAC and OAC) as well as gelation and viscosity. NSI is the solubilization measure of protein products in water solution at different pHs. WHC is the prevention of the ability of proteins to release water from their 3D structure (Haque et al. 2016). While NSI and WHC are usually determined using standard methods of AOCS method Ba 11–65 (AOCS 1998) and AACC method 88–04 (AACC 1983), respectively, no standard techniques have yet been developed for analysis of other functional properties.

WAC is an index representing water-binding properties of protein products. OAC is attributed to the capability of protein products to physically entrap oil through capillary attraction, and it's also associated with the availability of hydrophobic amino acid side chains on the surface of the protein molecules as primary sites to bind triglyceride chains (Kinsella and Melachouris 1976; Naczk et al. 1985; Sathe et al. 1982; Xu and Diosady 1994). The methods of Naczk et al. (1985) and Lin et al. (1974) were well adapted and modified by most researchers to analyze WAC and OAC.

The emulsifying property of protein products at the oil–water interface is expressed as emulsifying activity index (EAI), and the emulsion stability (ES) is expressed as a percentage of the emulsifying activity remaining after heating. Whip-ability is generally analyzed in terms of foam expansion (FE) and foam volume stability (FVS) values (Tabtabaei et al. 2017a, 2019). The methods of Yasumatsu et al. (1972) and Naczk et al. (1985) have been adapted for the determination of emulsifying properties, while the whipping test is mostly adapted from Patel et al. (1988) and Yasumatsu et al. (1972).

Food functional properties of air classified protein- and starch-rich fractions have been thoroughly studied. Pin-milled flours of eight different legumes, including chickpea, pea, northern, faba, field pea, lima, mung, and lentil were air classified by Sosulski and Youngs (1979) into protein-rich (fine) and starch-rich (coarse) fractions and their functional properties were compared with those of pin-milled lupine and defatted soybean flours having protein contents of 41.4% and 52.5%, respectively. Except for chickpea, air classification of all pin-milled legumes has significantly increased the protein content of the fine fractions to 47.7–66.6%, equivalent to 50–66.1% of the total protein, while the remaining coarse fractions contained only 12.2–15.6% protein. All air classified protein-rich fractions had higher OAC values of 81–94% than those of starting milled flours (63–78%) and air classified starch fractions (49–67%) but significantly lower than that of control lupine (125%) and defatted soybean (134%). All protein-rich fractions showed excellent emulsification properties, very close to the range of values reported for control lupine and defatted soybean as well as original milled flours, but significantly

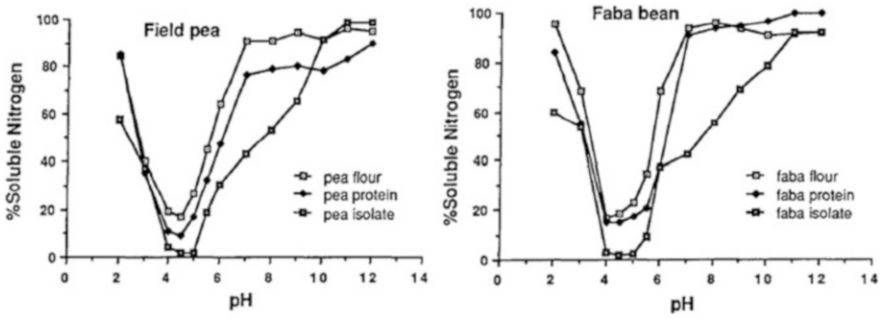

Fig. 2.2 Nitrogen solubility index (NSI) profiles for pin-milled flours, air classified protein fractions, and protein isolates of field pea and faba bean at different pHs of 2–12 (Sosulski and McCurdy 1987)

higher than those obtained for starch-rich fractions. Protein-rich fractions have also shown superior foaming properties compared to all tested fractions/products. Conversely, air classified starch fractions exhibited higher WAC, peak, and cold viscosities. Both protein and starch fractions showed superior gelation properties.

In another study, Sosulski and McCurdy (1987) investigated the air classification of pin-milled field pea and faba bean flours that resulted in doubling of the enrichment of the protein content in the protein fractions. Air classified protein-rich fractions were analyzed for WHC, OAC, emulsification, and foaming properties as well as NSI at different pH ranges of 2 to 12. Their functionality results were assessed according to the functional properties of faba bean and field pea protein isolates obtained by the wet isoelectric precipitation approach.

All products exhibited similar emulsification properties. Oil emulsification capacities (mL oil/0.1 g sample) of the air classified field pea and faba bean protein fractions were 37.2 and 35.7, respectively, very close to the capacities reported for field pea (34.6) and faba bean (34.6) flours as well as field pea (36.6) and faba bean (38.6) protein isolates. The WHC and OAC improved proportionally with the protein contents of the flours, air classified protein fractions, and protein isolates. For instance, the OAC values obtained at 21 °C for field pea flour, air classified protein fraction, and protein isolate were 41, 59, and 98%, increasing relative to their protein contents of 25%, 47.2%, and 80.3%, respectively. Interestingly, all protein fractions showed extremely high whip-ability and foam stability compared to other tested control flours and isolates. For instance, the initial foam volumes obtained after whip-ability of air classified field pea and faba bean protein fractions were 565 and 440 mL, respectively, significantly higher than those obtained for wet-fractionated field pea and faba bean protein isolates (315 and 200 mL, respectively).

Air classified protein fractions exhibited superior solubility profiles (Fig. 2.2) with very high NSI values on both sides of the isoelectric point of pH 4–5. Above isoelectric pH, field pea and faba bean protein isolates showed a gradual increase in NSI that might be associated with denaturation of isolates due to alkaline solubility,

acidic precipitation as well as drying impacts at high temperatures. This was not the case for air classified protein fractions as their NSI values were quickly recovered to over 80% at pH 7 and beyond. At isoelectric pH (4–5), protein isolates had close to zero NSI values, while for the air classified protein fractions, around 10–20% of the total nitrogen was soluble at this point. Similar NSI solubility patterns of the protein-rich fractions and pin-milled flours might represent the capability of the air classification process to retain the native bio-functionality of the protein products.

A double-pass approach of pin milling and air classification of roasted and non-roasted legumes including chickpea, navy, and pinto beans was investigated by Han and Khan (1990) with an analysis of the functional properties of both the protein- and starch-rich fractions in terms of WHC, NSI, and cold paste viscosity as well as emulsification and foaming properties. No significant functionality differences were reported for chickpea air classified fractions due to poor protein-starch separation efficiency. Pinto and navy bean starch fractions exhibited higher WHC, NSI, and cold paste viscosity, while their protein-rich fractions had excellent emulsification and foaming properties.

NSI profiles of the pin-milled chickpea, pinto, and navy bean flours and all their air classified protein fractions were similar to those obtained by Sosulski and McCurdy (1987) for field pea and faba beans. All fractions showed high acid (pH 2) solubility, followed by reduced solubility of ~10–20% at their isoelectric pH range (~4) that was aggressively increased to over 70% at pH 7. Dry-roasting heat treatment seemed to deteriorate the NSI and foaming properties of all fractions, while increasing the WHCs and cold viscosities that could be caused by denaturation and dissociation of protein molecules.

As can be seen, air classification processes are capable of producing various fractions not only with different compositional and nutritional properties, but also with various functional properties. Every food production system may require different ingredients with weak, intermediate, or strong functional properties, which would determine the desirability of the required additives or ingredients (Han and Khan 1990; Sosulski and Youngs 1979). For this purpose, air classification can be considered as a dry separation process to produce different functional fractions for a variety of food products.

Other recent studies were performed on determination of functional properties of lupine and other legume flours (Pelgrom et al. 2015b, 2014, 2013), representing the high solubility and improved foaming properties of the protein-rich fractions as well as high WHC and firm gelation properties of coarse starch-rich fractions. This will also emphasize on the fact that every fraction producing through air classification has its own utilizations and application in the food production systems.

Functional properties of the air classified lupine protein concentrate were assessed in terms of viscosity and foam stability (Pelgrom et al. 2014). Air classified lupine protein concentrate exhibited lower viscosity and higher foam stability than the starting lupine flour which might be beneficial for production of high-quality protein-rich beverages. The viscosity (Pa s) of lupine flour and air classified lupine protein concentrate was studied as a function of shear rate (s^{-1}). At tested shear rates of 1 to 100 s^{-1}, air classified lupine protein had significantly lower viscosity

compared to the lupine flour. For example, at the shear rate of 10 s^{-1}, lupine protein concentrate had ~100 times lower viscosity than the lupine flour. To assess their foam stability, the height of the foams prepared from lupine flour and air classified protein concentrate were recorded visually in terms of time (min). The time required for the foam from lupine flour to reduce its height from 12 to 0 cm was ~4 min, while that of the foam from air classified lupine protein was ~20 min. Heating of the air classified lupine protein fraction at 90 °C did significantly increase its viscosity while reducing its foam stability, possibly due to protein aggregation and denaturation.

Dry milling and air classification of yellow field peas were extensively studied by Pelgrom et al. (2013) who tested the effect of impact or jet milling at different intensities on effective dientanglement of starch granules from embedded protein particles. The classifier wheel speed of 4000 rpm was found as an optimized milling condition with minimal damage to the starch granules. The resulting milled flours were air classified at different classifier speeds to produce pea protein concentrates with 51–55% protein as well as starch-rich fractions with 10–20% protein. The WHC analysis of those fractions with less than 30% protein yielded two phases of pellets and supernatants where the WHC of the pellets increased by increasing protein content, similar to the results observed by Sosulski and McCurdy (1987) as well as Han and Khan (1990). The WHC analysis of pea fractions with over 30% protein yielded a single homogeneous solution containing ~26% protein, owing to the high solubility of the non-denatured proteins remained intact during air classification. Heating of the fractions formed gels with improved WHC and prevented the formation of homogeneous concentrates. The higher WHC could be attributed to starch gelatinization and reduced solubility of proteins as a result of denaturation caused by dissociation of pea protein subunits, revealing more water-binding sites (Abbey and Ibeh 1988; Damodaran 2008; Owusu-Ansah and McCurdy 1991).

While air classified pea protein concentrates with over 30% protein are excellent ingredients in production of high-protein beverages and liquid-type foods, they can also be used in the production of those foods that require significant WHC for structuring purposes such as meat analogues, pastes, or baked goods (Jia et al. 2021; Pelgrom et al. 2013).

Pelgrom et al. (2015b) explored further purification of the air classified pea protein concentrates through ultrafiltration (5 kDa regenerated cellulose membrane) of the top two layers obtained after aqueous extraction (20% w/w, room temperature and 30 min) and centrifugation (4500 rpm and 30 min). The resulting ultrafiltered native protein fraction contained 67% protein, equivalent to 63% of the total protein. The heat-induced gelatinization behavior of the ultrafiltered native protein concentrate was compared with those of air classified protein concentrate (~43% protein) and conventional pea protein concentrate (~72% protein). The ultrafiltered protein fraction made significantly stronger gel compared to the gel prepared using the air classified protein fraction since its gel network structure was less dispersed by starch granules or other cell wall components. However, the gel strength of the ultrafiltered protein was still lower than that of pea protein isolate, probably due to the high WHC of the isolate denatured proteins. The energy consumption of the air

classification-ultrafiltration technique is estimated about 6 times lower than that of the conventional wet processes (Pelgrom et al. 2015b). These results indicated the possibility of producing native protein concentrates with relatively strong gel strength as novel food ingredients through air classification as a main sustainable route.

2.6 Advantages, Disadvantages, and Limitations of Air Classification

One of the main advantages of air classification as a dry fractionation process is the lack of addition of solvents and chemical reagents, which is common in conventional wet fractionation processes. Dry fractionation preserves the native structure and functionality of the components. The harsh processing conditions in most wet fractionation methods can denature proteins and lower the functionality of the protein isolates. At the same time, protein-rich fractions obtained by air classification have shown better whip-ability, foam stability, and emulsification. These characteristics can then be utilized for further downstream processing, such as optimizing nutrition, texture, accessibility, and preservability to appease the consumer. Since air classification is a dry fractionation process, it does not create effluents for further treatment and disposal and reduces the chance of microbial contaminations compared to wet fractionation processes (Assatory et al. 2019; Jafari et al. 2016; Mondor et al. 2012; Tabtabaei et al. 2019, 2017b, 2016b, 2016a; Vitelli et al. 2021, 2020). Air classification is much more energy-efficient compared to traditional wet fractionation processes; while air classification could produce 55.8 g protein/MJ of energy, conventional wet processes produce approximately one-quarter of protein, i.e., 14.6 g protein/MJ (Schutyser et al. 2015).

Air classification has been accredited for organic food production, and their products are exempted from some of the standard codes such as E-numbers (Schutyser et al. 2015). Air classification also facilitates the production of functional hybrid ingredients containing protein and fibers (Silventoinen et al. 2021). There are, however, several disadvantages and limitations for air classification compared to conventional wet processes and tribo-electrostatic-based dry fractionations. Protein's purity, on average, is lower in air classifications and tribo-electrostatic separations than wet fractionations since some fine and coarse particles may go in the wrong fraction due to random physical factors such as air turbulence and inter-particle collisions. The success of air classification to produce protein concentrates relies highly on the successful disentanglement of plant cell contents through milling, which means the milling type and operating conditions could control the efficiency of the air classification process. Air classification could also fail to separate different particles (starch-, protein-, fiber-rich fractions) of similar size and density, and therefore wet fractionation or triboelectric separation processes are recommended. While disentanglement of protein and starch particles during the milling is the key to a successful separation in air classifiers, high milling speeds can damage the particles and therefore negatively affect the physical and functional properties of the fractions.

If milling is too coarse, the presence of protein aggregates, starch granules, and other cell components will interfere with air classification credibility. At the same time, fine milling (smaller particle size) to better disentangle the contents might lower the efficiency of the air classification process as small starch granules might become difficult to separate from protein bodies in the fine fractions (Delcour and Hoseney 2010). These factors suggest that the preceding milling step needs to be calibrated based on the raw material.

Single-pass mass yields of the protein-rich fractions are generally low in the air classification process. Therefore, multiple stages of classification are usually needed to produce a product with the desired quality, quantity, and purity. It is also important to note that a second classification step may increase the protein yield but generally at the cost of lowering its purity, i.e., the protein content.

Furthermore, the high fat content of some of the raw material may hinder dry processing due to lowered particle dispersibility or material adhesion to milling or fractionation equipment. Therefore, lipid-heavy feeds, such as soy and lupine, may need to be defatted prior to the milling and air classification processes. As a result, the pre-processing and defatting step, maintenance, such as regular cleaning, and their associated costs must be taken into consideration once dealing with high fat feeds. It is also important to note that the pre-treatment might be more costly and increase the operation timeline.

The efficiency of the milling process i.e., disentanglement of protein and starch bodies as well as the air classification process also depends on the moisture content. Lower moisture, in general, is favored as it makes the feed more brittle, and milling becomes easier. However, lower moisture content for some materials, such as pea and lupine, could also mean reduced protein purity in the fine fraction. High moisture content also negatively affects the efficiency of the fractionation process due to the agglomeration of the starch-, fiber-, and protein-rich particles. Furthermore, the protein bodies, for example in lupine and pea flour, might dissolve during pre-soaking, which lowers the efficiency of the air classification by reducing their particle density (Pelgrom et al. 2015c, 2014, 2013).

2.7 Tribo-Electrostatic Separation, an Alternative Dry Fractionation Technology

Tribo-electrostatic separation (TES) technology has been recently explored as an alternative dry separation method to air classification.

One of the similarities between TES and air classification is that they both required dry milling steps prior to the separation process (Assatory et al. 2019), and the research has shown that the type and intensity of the milling primarily affect the plant protein purity and its separation efficiency (Pelgrom et al. 2013; Vitelli et al. 2020). Therefore, the challenges faced with dry milling of agro-materials prior to air classification can also be applied to TES processes since the disentanglement of protein particles from other cell materials is still necessary prior to effective tribo-charging and electrostatic separation.

The separation mechanism in air classification processes depends mainly on particle size and density (Day 2013; Pelgrom et al. 2013). However, in TES applications, the separation mechanism is based on the tribo-charging behavior of milled particles (Assatory et al. 2019; Konakbayeva and Tabtabaei 2021; Tabtabaei et al. 2016a). Tribo-charging is a complex phenomenon that could be affected by particles' surface compositions, physicochemical properties, and environmental conditions (i.e., temperature and relative humidity). In addition, tribo-charging depends on the contact material, size, and shape of the charging apparatus as well as gas flow rate, intensity, and direction of impacts (Konakbayeva and Tabtabaei 2021; Mayr and Barringer 2006; Mehrtash et al. 2022; Tabtabaei et al. 2016a; Wang et al. 2015, 2014; Xing et al. 2021).

During TES-based processing, the milled flour particles are first directed inside the charging apparatus utilizing gas flow and tribo-charged by physical contact with themselves and the enclosing surface of the charging apparatus system. The charged particles are then separated from each other under the influence of an external electric field (Assatory et al. 2019). Preliminary tribo-charging studies of single-component agro-materials with polytetrafluoroethylene (PTFE) revealed that protein particles, due to their ionizable functional groups, can acquire substantially different charge-to mass-ratios compared to starch granules and dietary fiber particles (Mayr and Barringer 2006; Tabtabaei et al. 2016a). Therefore, the charged protein-rich particles are attracted toward the electrode plate, where most starch and dietary fiber particles are repelled. Tribo-charging can be performed in different charging apparatuses, including fluidized bed, tribo-charging tube (Konakbayeva and Tabtabaei 2021; Tabtabaei et al. 2016a), and/or charging slit (Wang et al. 2015). The contact material of the tribo-charging apparatus plays a vital role in the effective separation of protein particles from non-protein particles.

Some studies found PTFE as an effective material to differently charge protein and starch granules so that they can be separated from each other under electric fields during TES of milled starch-rich legumes (Tabtabaei et al. 2016a, b). In other studies, aluminum was also an effective material for protein separation of milled defatted non-starch legumes, such as lupine (Pelgrom et al. 2015c). Different tribo-electrostatic separators are custom-designed, consisting of the tribo-charging apparatus and an electrostatic separation chamber equipped with electrode plates and a high voltage supply. The electrostatic separation chamber could be designed vertically (Tabtabaei et al. 2016a, b; Wang et al. 2015; Xing et al. 2021) or horizontally (Pelgrom et al. 2015c; Wang et al. 2014); however, studies showed that the vertical separation chamber is more effective in plant protein enrichment. The schematic diagram of a tribo-electrostatic separator is given in Fig. 2.3, consisting of a fluidized bed to disperse milled flour particles, a tribo-charging tube, and a vertical separation chamber equipped with two electrode plates (Konakbayeva and Tabtabaei 2021).

TES has been recently applied to fractionate plant proteins from various agricultural materials, including oilseed meals (Basset et al. 2016; Pelgrom et al. 2015c; Wang et al. 2016b; Xing et al. 2018), legumes (Jafari et al. 2016; Pelgrom et al. 2015c; Tabtabaei et al. 2017c, 2016a, b; Vitelli et al. 2021; 2020), cereal brans (Hemery et al. 2011; Sibakov et al. 2014; Wang et al. 2016a), and cereal groats

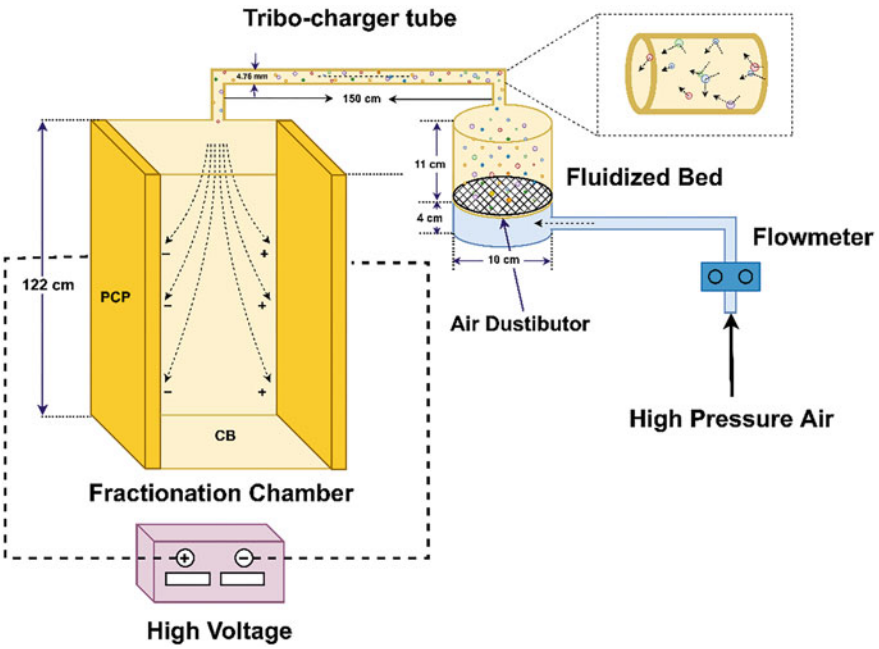

Fig. 2.3 Schematic diagram of the custom-built tribo-electrostatic separator (Konakbayeva and Tabtabaei 2021)

(Konakbayeva and Tabtabaei 2021). Recent studies showed the effectiveness of TES in protein enrichment of starch-rich legumes, non-starch legumes, and cereal brans. The protein enrichment levels were not significant in oilseed meals and cereal groats. One of the main reasons limiting adequate protein enrichment in cereal groats is the presence of large bran particles, preventing the effective fluidization and chargeability of the milled groats. For the case of oat groats, a sieving step was initially applied to physically remove large protein-rich bran particles before applying TES to starchy endosperm-rich sieved fractions (Konakbayeva and Tabtabaei 2021).

TES has also been applied as a post-treatment approach to air classified fractions to improve further the protein purity and separation efficiencies obtained by air classification (Pelgrom et al. 2015c; Xing et al. 2020).

Additionally, some studies have been performed on the effect of TES operating conditions on plant protein enrichment level, including gas flow rate, plate voltage, size (i.e., diameter and length) of the tribo-charger, and the placement of the electrode plates (i.e., plate angle and distance between electrode plates) (Tabtabaei et al. 2016b; Wang et al. 2015). Some studies have also been performed on electrode plate fouling and its effect on protein enrichment. The distribution of charged protein particles across the top, middle, and bottom of the vertical electrode plate was studied at different laminar and turbulent gas flow rates (Tabtabaei et al. 2017c).

Similar to air classified protein fractions, the functional food properties of the tribo-electrostatically separated protein fractions showed better solubility, emulsification, and foaming properties compared to the protein concentrates/isolates produced by a wet process (Tabtabaei et al. 2019). In addition, the protein-enriched fractions through TES processes contained higher sulfur-containing amino acids and albumin proteins than the wet-fractionated proteins, resulting in improved solubility and foaming properties (Jafari et al. 2016).

While tribo-electrostatic separation has proven to be an economically viable, easy-to-set-up, and effective process for the plant protein enrichment of various agro-materials, there are still some fundamental gaps, particularly the tribo-charging behavior of agricultural compounds under different operating and environmental conditions. Other challenges to overcome include understanding the electrostatic separation behavior of the charged particles under gas turbulence and the role of inter-particle collisions in the separation chamber under gravity and electrostatic forces. Other studies should also be performed to scale the TES approach and its safe operation.

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Barometric Membrane Technologies for Plant Protein Purification

3

Alfredo Cassano and Carmela Conidi

Abstract

Proteins, hydrolysates and peptides from both animal and vegetable sources exhibit specific biological activities, which may have effect on functional or pro-health properties of food products. Among the available technologies, membrane filtration is one of the most sustainable and cost-effective technique for the recovery and purification of protein-based compounds. This chapter provides a comprehensive overview on the use of barometric membrane processes, also in integrated systems, for the recovery of protein-based compounds from different sources (cereals, oilseeds, microalgae, soy, agro-food by-products, among others) highlighting typical advantages and limitations over competitive techniques.

Keywords

Pressure-driven membrane operations · Proteins recovery · Vegetable sources · Soy proteins · Agro-food by-products.

Abbreviations

APC	Allophycocyanin
CA	Cellulose acetate
CPC	C-phycocyanin
DF	Diafiltration
EC	Emulsifying capacity

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MBR	Membrane bioreactor
MF	Microfiltration
MPH	Mushroom protein hydrolysate
MWCO	Molecular weight cut-off
NF	Nanofiltration
PAN	Polyacrylonitrile
PES	Polyethersulphone
PP	Polypropylene
PS	Polysulphone
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
RC	Regenerated cellulose
RO	Reverse osmosis
SPC	Soy protein concentrate
SPI	Soy protein isolate
TMP	Transmembrane pressure
UF	Ultrafiltration
VCR	Volume concentration ratio
VRF	Volume reduction factor

3.1 Introduction

Among various natural polymers, plant proteins, including soy protein, zein, wheat gluten, are attracting growing attention in both scientific research and industries owing to their easy accessibility, low cost and antioxidant potential (Kumar et al. 2008). In addition, bioactive peptides and amino acids isolated from many plant sources and fruit processing wastes have been found to possess antimicrobial, antihypertensive and anti-inflammatory properties (Banerjee et al. 2017; Meneguetti et al. 2017). Their advantages in terms of low molecular weight, simple structure, easy adsorption and stability under different conditions have drawn the attention of several researchers towards the extraction and recovery of these compounds aimed at developing functional ingredients. On the other hand, the substitution of synthetic antioxidants by natural ones is gaining interest due to the consumers' preferences and health concerns associated with the use of synthetic food additives.

The recovery of valuable biomolecules from natural sources, such as plants, food by-products or even algae and microalgae requires multiple steps in relation to the complexity of the raw material, the sensitivity of the target compounds to the processing conditions and the safety and specifications of the final product. It is typically conducted through the so-called 5-Stages Universal Recovery Process which includes (i) macroscopic pre-treatment, (ii) macro- and micro-molecular separation, (iii) extraction, (iv) purification and (v) product formation (Galanakis 2012). Among these steps, extraction is the most important ones and numerous

conventional and non-conventional techniques have been assayed towards the properties of the target components and the respective bioresources. The traditional extraction methods used to obtain these compounds have several drawbacks since they employ large amounts of toxic solvents. In addition, they are time consuming, laborious, have low selectivity and low extraction yields.

Supercritical fluid extraction (SFE) and sub-critical water extraction (SWE) provide several operational advantages over traditional extraction methods due to their higher selectivities, shorter extraction times and use of nontoxic, noninflammable and noncorrosive solvents, in line with the label 'natural' (Essien et al. 2020).

Pressurized liquid extraction (PLE), enzymatic-assisted extraction (EAE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) have been also proposed in order to increase the efficiency and rate of extraction while maintaining the bioactivity properties of sensitive target compounds (Plaza and Turner 2015; Oreopoulou and Tzia 2007).

The purification stage aims at the isolation or the clarification of the target compounds from co-extracted impurities. In this context, membrane technologies offer potential sustainable solutions for the recovery of these compounds from complex streams without affecting their structure and function, which ultimately translates into their bioactivity. The reason for the fast and rapid increase of membrane systems in food processing industry is mainly related to their typical advantages over conventional technologies such as: high selectivity, easy scale-up, modularity, low operating temperature with minimization of thermal damage, gentle product treatment, no phase change and use of chemical additives, low energy consumption (Li and Chase 2010).

Among the membrane processes which have been intensively developing in the recent years, baromembrane processes hold a special place in relation to their use and application in a wide range of industrial sectors.

This chapter will focus on the potential of pressure-driven membrane operations in the recovery of protein-based compounds from natural sources and agro-food by-products. It aims to provide a comprehensive information on basic principles of the technology and its impact on the recovery of protein-based compounds from different sources (cereals, oilseeds, microalgae, soy, agro-food by-products, among others) highlighting typical advantages and limitations when compared to other conventional techniques.

3.2 Barometric Membrane Processes: Basic Principles

Barometric membrane processes are based on the use of permselective barriers through which solvent fluids with permeable solutes are selectively transported under a hydrostatic pressure applied on the feed side. As a result, the feed solution is divided into a permeate fraction containing all components which have permeated the membrane and a retentate fraction containing all rejected compounds, within some of the solvent.

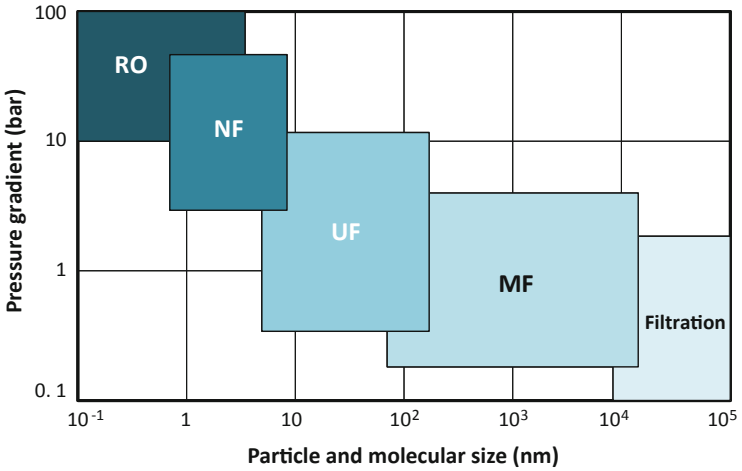

Fig. 3.1 Separation capabilities of barometric membrane separation processes

These processes are classified into four categories which include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), according to the membrane pore size, the required transmembrane pressure (TMP) and the separation mechanism (Van der Bruggen et al. 2003). In Fig. 3.1 a schematic of the separation capability of pressure-driven membrane processes is shown.

MF membranes generally have a symmetric structure and pores with diameter from 0.1 to 10 μm. Such membranes retain dispersed particles such as colloids, fat globules, or cells: these particles are generally larger than those separated by UF and RO. Since only large particles are separated by the membrane the osmotic pressure difference between the feed and the filtrate solution is negligible; therefore, hydrostatic pressure differences used in MF are relatively small (in the range of 0.5–2 bar).

UF membranes are typically asymmetric in structure with a dense active layer of 0.5–1 μm in thickness supported by a more porous support layer of greater thickness. Pore sizes in the skin layer are in the range 5–100 nm. Typical rejected species include biomolecules, polymers and colloidal particles, as well as emulsions and micelles (Charcosset 2012). These membranes are characterized by the molecular weight cut-off (MWCO), defined as the equivalent molecular weight of the smallest species that exhibit 90% rejection. The MWCO for UF membranes ranges between 1 and 1000 kDa. Hydrostatic pressures required decrease with increasing MWCO and are approximately between 2 and 8 bar.

The separation capabilities of NF membranes are situated between those of UF and RO membranes, with pore size typically of 0.5–2 nm, corresponding to MWCO of 200–1000 Dalton. These membranes are often negatively charged so that the anionic repulsion mainly determines the salt rejection: as a result monovalent ions are less retained than multivalent ones (higher the ionic charge, greater the saline rejection). Operating pressures are in the range 3–30 bar (Paul and Jons 2016).

RO membranes are generally used to separate low molecular weight compounds from a relatively pure solvent. The pore size range for RO applications is between 0.1–1 nm and solutes with molecular weight greater than 300 Dalton are separated. Operating pressures applied in RO are in the range 10–100 bar. Water desalination, brackish water and wastewater treatment, as well as food concentration, are well-established applications of RO.

All these processes are usually operated in a cross-flow configuration: the feed stream flows tangentially to the membrane surface at a certain velocity to minimize cake formation and hence membrane fouling of the membrane. This configuration is self-cleaning if compared to the traditional dead-end configuration in which the feed stream flows perpendicularly to the filter media.

Materials for fabrication of commercial membranes include synthetic polymers, ceramics, inorganics and metals. Among them, polymeric membranes dominate the industrial market of pressure-driven membrane operations. The most common polymeric membranes are manufactured from polysulphone (PS), polyethersulphone (PES), polytetrafluoroethylene (PTFE), polypropylene (PP), polyethylene (PE), polyvinylidene fluoride (PVDF), polyacrylonitrile (PAN), polyamide (PA), cellulose acetate (CA), regenerated cellulose (RC) and cellulose derivatives. Ceramic membranes are manufactured from inorganic materials (e.g. alumina, zirconia, titania and silica). They exhibit high resistance to aggressive media (acids, alkalis, strong solvents) and high mechanical and thermal stability. Although their production costs are higher than those of polymeric membranes, they are ecologically friendly, durable and have a longer lifetime. On the other hand, high capital costs (3 to 6 times more than polymeric membranes) have limited the widespread acceptance of these membranes.

For continuous membrane operations, membranes are installed in proper devices known as membrane modules. On large industrial scale, membrane modules are available in five basic designs: hollow fibre, spiral-wound, tubular, plate and frame and capillary. They are quite different in their design, mode of operation, production costs and energy requirement for pumping the feed solution through the module.

The selection of a proper configuration for a specific application depends on different parameters such as raw material specifications, final product desired properties, the production costs, packing density, energy consumption and especially the control of concentration polarization and membrane fouling.

Membrane fouling is a major operating problem of pressure-driven membrane operations. It can be considered a long-term flux decline caused by the interaction of chemical species and their deposition on the membrane surface or within the membrane pores. Several mechanisms can contribute to membrane fouling including an increased interfacial concentration due to concentration polarization, the adsorption of solutes within the pores, a partial or complete pore blocking, the formation of a cake from rejected solutes and the precipitation or gelation of inorganic and organic particulates at the membrane surface (Schäfer et al. 2000).

The performance of pressure-driven membrane operations is expressed in terms of permeate flux and separation properties. They are a function of the membrane

permeability to different compounds in the feed solution, the operating conditions (temperature, cross-flow velocity, TMP) and the process design.

The permeate flux is assessed by measuring the filtrate (permeate) throughout per unit membrane area and time that passes through the membrane:

$$J_p = \frac{V_p}{A \cdot t} \tag{3.1}$$

where J_p (L/m^{2}h) is the permeate flux, and V_p the volume of permeate (L) collected in a certain time t (h) through the membrane surface area A (m^2).

The membrane rejection (R) coefficient reflects the membrane selectivity and can be calculated for each solute as:

$$R = \left(1 - \frac{C_p}{C_f}\right) \cdot 100 \tag{3.2}$$

where C_p is the solute concentration in the permeate and C_f the solute concentration in the feed. Rejection values are between 0% (for solutes having highest probability to pass through the membrane) and 100% (when solutes are completely retained by the membrane).

The volume reduction factor (*VRF*) or volume concentration ratio (VCR) is defined as the ratio between the initial feed volume and the volume of the resulting retentate given by:

$$VRF = VCR = \frac{V_f}{V_r} = 1 + \frac{V_p}{V_r} \tag{3.3}$$

where V_f, V_p and V_r are the volume of feed, permeate and retentate, respectively.

3.3 Recovery of Protein-Based Compounds from Vegetable Sources

The production of soy proteins is one of the most important activities of the agro-food sector accounting for 69% of global plant protein consumption in the world. These proteins are widely used to formulate foods with the goal of improving their nutritional and/or functional qualities. They are composed of a mixture of albumins and globulins, 90% of which are storage proteins with globular structure (Tian et al. 2018).

Typically, three kinds of commercial soy protein products are processed from soybean: soy flour (SF), soy protein concentrate (SPC) and soy protein isolate (SPI).

SPCs and SPIs are produced at the industrial scale by isoelectric precipitation. This process has a high productivity, however, it also generates large volumes of effluent.

UF is a valid alternative to the use of isoelectric precipitation for the production of soy protein isolates or concentrates from soy protein extracts. The use of UF allows

the recovery of all solubilized proteins avoiding the formation of whey-like products resulting in an increased protein recovery (Nichols and Cheryan 1981). Undesirable compounds such as oligosaccharides (sucrose, raffinose and stachyose) and phytic acid can be selectively separated from the proteins through a selection of optimal operating parameters and membrane types (Kumar et al. 2003).

SPIs with low phytic acid level were produced by combining bipolar membrane electrodialysis (BMED) and tangential flow UF/DF (Ali et al. 2010). The combination of a 100 kDa PS membrane with DF resulted in high phytic acid removal and high levels of protein purification. Phytic acid removal was affected by pH: high removal percentages were observed within a pH range of 5–6.7.

A combined UF/DF system was also investigated by Shallo et al. (2001) to concentrate soy proteins from defatted soy flour enzymatically treated with commercial pectinases. The concentrated product consisted of 78.5% protein and had reduced levels of phytic acid. In particular, proteins with molecular weights greater than 6.5 kDa were rejected by the UF membrane and retained in the soy concentrate. Protein recovery yields resulted from 17% to 26% higher than those achieved with conventional commercial processes.

According to Skorepova and Moresoli (2007) UF membranes with MWCO of 50 kDa represent the best option to obtain high values of permeate fluxes, high protein rejection and high removal levels of oligosaccharides. The use of UF membranes with MWCO between 5 and 30 kDa allows to retain isoflavones, widely recognized for their anticarcinogenic properties, due to their complexation with proteins (Singh 2007). Adversely, in the conventional isoelectric precipitation processes most of the isoflavones remain soluble after the precipitation step and are lost in the effluent.

Functional properties of SPIs obtained by a combination of UF with DF, including solubility, ability to emulsify, ability to bind water or fat and ability to form foams or gels resulted higher than those obtained with the traditional isoelectric precipitation processes. In particular, foam viscosities of membrane isolated soy products were in excess of 300,000 cps as compared to 33,000 cps for the commercial isolate (Manak et al. 1980).

SPIs can also be used as a source of peptides of interest for nutraceutical applications. In this context, UF membranes compete with other technologies such as chromatography and ion-exchange resins for the fractionation and purification of specific peptides. In particular, soy protein hydrolysates can be submitted to a sequential treatment with UF membranes of increasing MWCO value (i.e. from 5 to 100 kDa) in order to produce different soy peptide fractions (Deeslie and Cheryan 1991).

Roblet et al. (2012) analysed the fractionation of SPI hydrolysates with PES UF membranes in hollow fibre and spiral-wound configuration. Experimental results indicated that membrane configuration and treatment time influenced fractions composition, and consequently, their potential bioactive properties which in turn depend on their amino acid composition. Permeate fractions presented higher antioxidant activity in comparison with the retentates of both membranes. These phenomena were attributed to the presence in the permeates of small molecular weight

peptides under 1000 Da rich in tyrosine, phenylalanine and leucine residues or simply amino acids residues alone, released by both pepsin and pancreatin treatments.

RC UF membranes with MWCO of 1 and 3 kDa were investigated to recover proteins from the skim fraction obtained through an enzyme-assisted aqueous extraction of soybean (Campbell and Glatz 2010). The UF process with the 3 kDa membrane produced a protein concentration in the retentate between 55% and 70%, while reducing stachyose content from 6% to 2%. The overall protein yield was of 60%, similar to that of conventional SPC and SPI processes. UF with a 1 kDa membrane increased protein retention, but without reduction in stachyose.

Razavi et al. (1996) analysed the fouling mechanisms of UF membranes used in the treatment of aqueous extracts of soy flour. The thickness of the foulant deposit was approximately 0.2 and 0.4 μm for 50 kDa and 100 kDa membranes, respectively. The foulant deposit had pseudoplastic and viscoelastic properties and consisted of lipids in a globular form of 0.2 to 1 μm diameter. The recovery of the water permeability of the fouled membranes was achieved by a four-stage cleaning procedure including washing with sodium hydroxide, protease detergent, sodium hypochlorite and flushing with water.

Noordman et al. (2003) found that suspended solids mainly represented by insoluble milled bean material (mean particle size 25 μm) can have a positive effect on the permeate flux of defatted soy flour extracts treated with UF with tubular PS membranes of 100 kDa. As a result, a much higher concentration can be achieved in extracts with suspended particles compared to particle free extracts. In addition, the particles have also a positive effect on the reversibility of fouling.

Pea proteins, mainly represented by albumins and globulins, are an interesting alternative for soybean proteins which are the most represented plant protein isolates in the global market. Pea protein concentrates and isolates are valuable functional ingredients widely used in food formulations (Dhaliwal et al. 2021). They may be produced by air classification and alkaline or acid extraction (wet processes) (Sumner et al. 1981).

Mession et al. (2012) evaluated the use of UF membranes to extract pea proteins from defatted pea flour as alternative to acidic precipitation. The UF process was carried out by using 100 kDa PES membranes in flat-sheet configuration at 20 °C up to a VCR 3 followed by a continuous diafiltration up to a volume permeated ratio (VPR) of 3. Diafiltrate was quickly frozen and freeze-dried. The protein content increased from 74.6 wt% in the extract to 84 wt% in the concentrate and finally reached more than 95 wt% in the diafiltrate. The laboratory-prepared isolates had protein contents of 85.8 wt% (in comparison with 85.1 wt% of isolates obtained by acidic precipitation).

Similarly, Wiege et al. (1993) used UF and diafiltration with a plate and frame process unit as alternative to isoelectric precipitation for the isolation of pea protein from an aqueous extract of wrinkled pea flour followed by centrifugation. In optimized conditions of temperature, pressure and degree of concentration, a crude protein content of 82 wt% was achieved in the concentrate. The yield of the native final product was 83%.

UF membranes can be also used to separate peptide fractions from pea protein hydrolysates. Peptides with inhibitory activities against trypsin and chymotrypsin were isolated by Awosika and Aluko (2019a) after hydrolysis of pea protein with proteases. In particular, yellow field pea protein concentrate was hydrolysed using alcalase, pepsin, trypsin and chymotrypsin. The digestion mixture was then centrifuged and the supernatant was fractionated into different peptide sizes in a sequential manner by using a UF system fitted with 1, 3, 5 and 10 kDa membranes. First, the supernatant was filtered through the 1 kDa membrane producing a permeate fraction of <1 kDa. The retentate was mixed with an equal amount of water and then filtered through a 3 kDa membrane to obtain a 1–3 kDa permeate. Similarly, retentates of 3 kDa and 5 kDa membranes were filtered through 5 kDa and 10 kDa membranes producing permeate fractions of 3–5 kDa and 5–10 kDa. The <1 kDa peptide fraction showed a slightly stronger affinity to trypsin when compared to the unfractionated trypsin hydrolysate. On the other hand, the unfractionated chymotrypsin hydrolysate had greater affinity for chymotrypsin when compared to the 1–3 kDa peptide fraction. A similar approach was used by Awosika and Aluko (2019b) to produce yellow field pea protein-derived peptides as inhibitors of α-amylase, α-glucosidase and pancreatic lipase activities.

Mushrooms are a great source of nutritionally valuable compounds, including protein and phenolic antioxidants (Rosello-Soto et al. 2016). The fractionation of water mushroom extracts by UF was investigated by Cheung and Cheung (2005). In particular, mushroom samples (*Lentinus edodes* and *Volvariella volvacea*) were extracted sequentially with petroleum ether, ethyl acetate and methanol. The methanol-insoluble residues were extracted with boiling water and then fractionated by using a 10 kDa UF membrane producing two different subfractions containing high molecular weight (HMW) and low molecular weight (LMW) compounds. The protein content of the LMW subfraction for L. *edodes* and V. *volvacea* after UF was 28.7% and 22.6%, respectively, and resulted significantly higher than that of the HMW subfraction (3% and 6%, respectively). This fraction was highly active against lipid peroxidation of rat brain homogenate.

Mushroom protein hydrolysates (MPHs) of *Agaricus bisporus* obtained with single and sequential enzymes were fractionated into peptides of various molecular weights by using a combination of UF membranes with MWCO of 1, 3, 5 and 10 kDa (Kimatu et al. 2017). Starting from the 1 kDa membrane, each retentate was the feeding solution of the next UF step (Fig. 3.2). Hydrolysate yields and protein recoveries were higher than 57% and 43%, respectively. The highest antioxidant activity measured with the ferric reducing antioxidant power (FRAP) test was detected in fractions from alcalase and pancreatin obtained with 1 and 3 kDa membranes.

The whole results suggested the high potential of both hydrolysates and retentate fractions as bioactive ingredients for use in the formulation of functional foods as well as natural antioxidants in lipid food systems.

Recently, Labus et al. (2020) investigated the use of MF and UF membranes for the purification of mushroom tyrosinase as alternative to the common procedure based on dual successive salting out followed by centrifugation. Among the MF

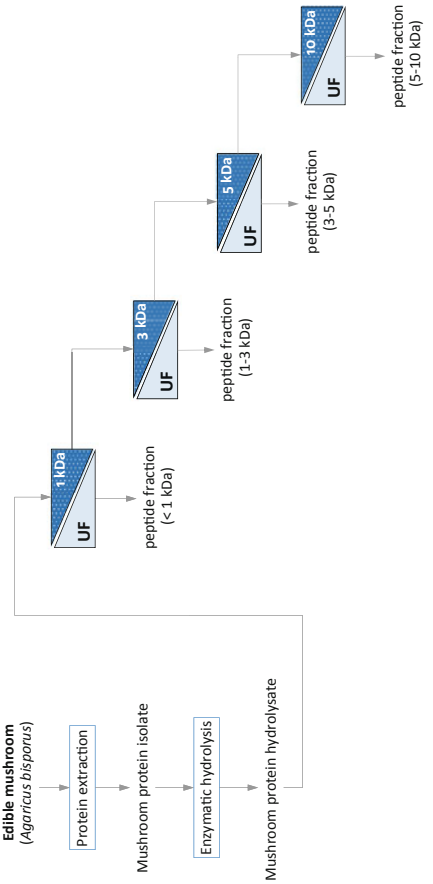

Fig. 3.2 Schematic of mushroom protein fractionation by UF membranes (adapted from Kimatu et al. 2017)

membranes investigated for the removal of undesired high molecular weight compounds, a nitrocellulose membrane was selected due to high recovery of enzymatic activity. The MF permeate was submitted to a concentration/diafiltration step with a PES membrane of 300 kDa. This process allowed to produce tyrosinase preparations with a reasonable purification level (recovery of 8% of proteins and 58% of tyrosinase activity) and the higher thermal stability than in the case of salting-out procedure.

The fractionation of protein hydrolysates by membrane operations is an attractive approach to improve the potential of proteins with poor functional and sensory properties, making them suitable for applications as food additives, nutritional therapies or pharmaceutical ingredients. Xie et al. (2008) investigated the fractionation of alfalfa leaf protein hydrolysates with a UF membrane system equipped with a 3000 Da PS membrane. The purified peptides, represented mostly by low molecular weight (<1000 Da) peptides, showed high antioxidant activity, nutritive value, chelating ability and reducing power.

Potato proteins are a promising source for the production of bioactive compounds as materials for developing functional foods with a positive impact on cardiovascular health. UF membranes with MWCO of 3, 5 and 10 kDa were used to remove enzymes and not-hydrolysed proteins from hydrolysates of protein isolates and by-products from the potato industry obtained by treatment with alcalase and esperase (Pihlanto et al. 2008). Hydrolysis increased the inhibition of the angiotensin-converting enzyme (ACE) and the radical-scavenging activity. All selected membranes produced a permeate fraction containing ACE-inhibitory compounds. The scavenging capacities of alcalase and esperase hydrolysates (52% and 40%, respectively) increased in the retentate and decreased in the permeate fraction of the 3 kDa membrane. In particular, the radical scavenging of retentate and permeate fractions of alcalase hydrolysates were of 63% and 44%, respectively. For retentate and permeate fractions of the esperase hydrolysate values of 89% and 24% were measured, respectively.

Hydrolysates obtained from wheat gluten (a by-product of wheat starch production) by enzymatic hydrolysis with papain were fractionated by using 5 kDa UF membranes (Wang et al. 2007). The resulting permeate and retentate fractions included 26.4% and 53.8% of protein. These fractions showed higher surface hydrophobicity at pH 7.0 ($H_0 = 324.1 \pm 26.5$ and 295.6 ± 23.7, respectively) in comparison with the hydrolysate ($H_0 = 287.5 \pm 16.3$). Their antioxidative activities, measured by linoleic acid and DPPH tests, were also higher in comparison with the hydrolysate. The highest antioxidative activity was found in the permeate fraction which exhibited significant ($P < 0.05$) inhibition of linoleic acid peroxidation.

Rapeseed protein isolates from oil industry waste were prepared by UF through the use of PS membranes with a MWCO of 10 kDa according to the procedure depicted in Fig. 3.3. After processing, the protein isolates contained 98.7% of protein and 1.2% of fat. They showed good solubility (52.5–97.2% in a pH range of 3–9) as well as higher emulsification capacity (693 ml oil/g protein) and emulsification stability (96%) in comparison with those of precipitated protein isolates (693 ml oil/g protein and 8.5%, respectively). These properties can be exploited for replacing

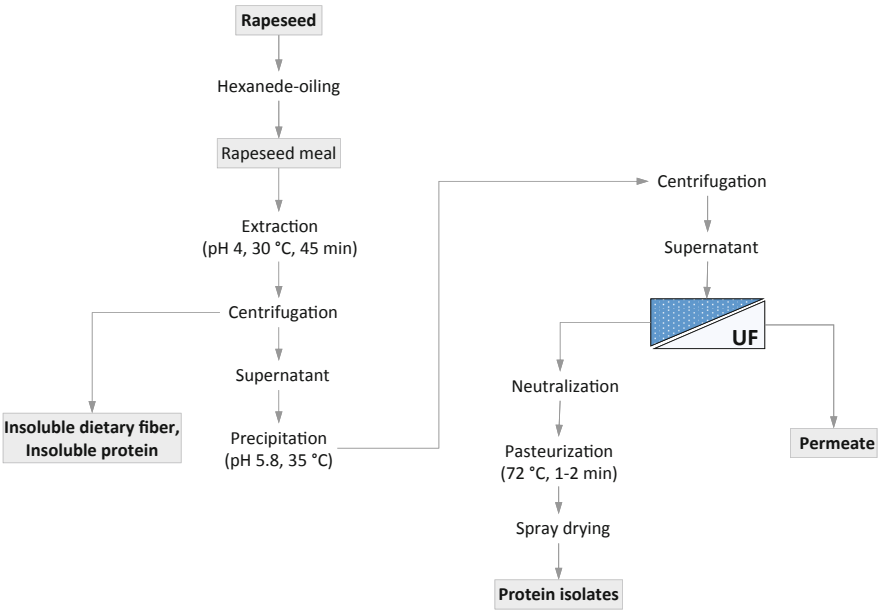

Fig. 3.3 Protein extraction process from rapeseed meal (UF, ultrafiltration) (adapted from Yoshie-Stark et al. 2008)

animal proteins in food production (i.e. as a replacement for egg white for allergic people) (Yoshie-Stark et al. 2008).

3.4 Recovery of Protein-Based Compounds from Microalgae

Microalgae represent a promising renewable feedstock of healthy food ingredients and functional food products due to their high content of valuable bioactive compounds, such as vitamins, essential amino acids, polyunsaturated fatty acids, minerals, carotenoids, enzymes and fibres (Matos et al. 2017). Compared to other natural sources of bioactive ingredients, microalgae have many advantages including a wide biodiversity, the possibility to grow under conditions of limited water consumption and the flexibility of their metabolism, which could be adapted to produce specific molecules (Buono et al. 2014).

Microalgae-based protein products can be classified, on the basis of their protein content and the degree of refining, as whole-cell protein, protein concentrates, isolates, hydrolysates and bioactive peptides (Soto-Sierra et al. 2018). Their production involves microalgae cultivation (upstream) followed by harvesting, drying, cell disruption, protein extraction, hydrolysis and separation (downstream processing) (Fig. 3.4). In this context, membrane-based operations, and especially UF, represent useful approaches in both upstream and downstream processing steps. Indeed, they can be used for microalgae harvesting (as alternative to centrifugation and

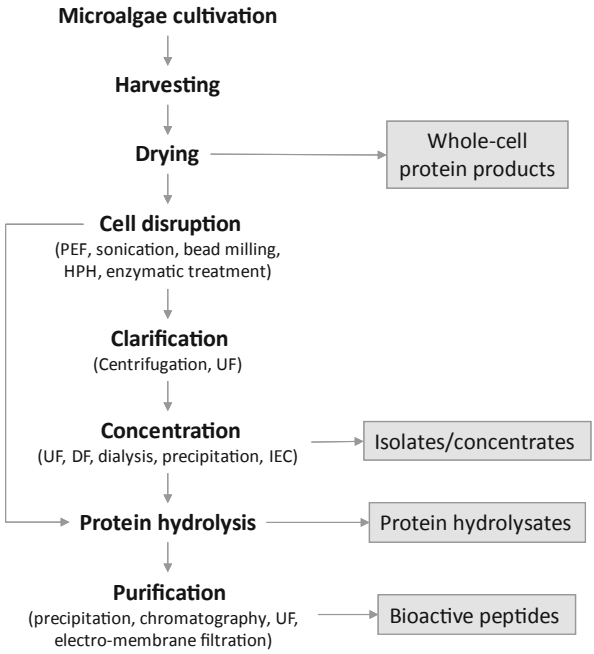

Fig. 3.4 Upstream and downstream processing steps for the production of protein-based compounds from microalgae (PEF, pulsed electric field; HPH, high-pressure homogenization; UF, ultrafiltration; DF, diafiltration; IEC, ion-exchange chromatography) (adapted from Soto-Sierra et al. 2018)

flocculation-assisted settling) but also in the production of protein concentrates and isolates as well as in the production of small peptides with bioactivity potential.

A PES UF membrane of 300 kDa was used to concentrate proteins of *Chlorella vulgaris* after solubilization with high-pressure cell disrupter under pH 7 or 12 followed by centrifugation of the microalgae suspension (Ursu et al. 2014). The majority of the proteins were recovered in the retentate (87% and 95% for pH 7 and 12, respectively) and were mainly represented by complex macromolecular aggregates with a molecular weight above 670 kDa, independently from the pH. On the other hand, the permeate stream contained proteins with molecular weights ranging between 1.4–9.2 kDa at pH 12.

The analyses of the emulsifying capacity (EC) revealed that proteins obtained by precipitation presented lower EC than those obtained by UF. In addition, the proteins from permeate showed a higher EC than the proteins from retentate (3740 mL oil/g protein in permeate samples versus 2310 mL oil/g protein in retentate samples after extraction at pH 7). This behaviour was attributed to a denaturation of proteins during extraction leading to formation of aggregates recovered in the retentate; adversely, only native proteins with emulsifying properties were found in the permeate.

Kulkarni and Nikolov (2018) investigated a process for the selective extraction of carotenoids and chlorophylls from *Chlorella vulgaris* biomass, followed by alkaline extraction of proteins and their fractionation and concentration by two-stage tangential UF. Pigments were first extracted with ethanol from the wet, freeze-thawed biomass after which the cells were subjected to complete lysis using high-pressure

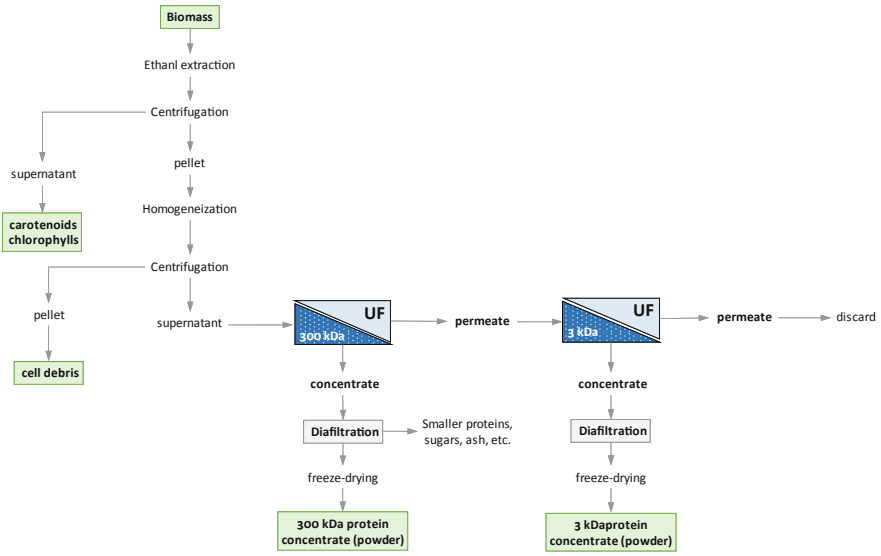

Fig. 3.5 Selective extraction of pigments and functional proteins from Chlorella Vulgaris (UF, ultrafiltration) (adapted from Kulkarni and Nikolov 2018)

homogenization for release of proteins followed by clarification of the cell lysate by centrifugation. The clarified extract previously was ultrafiltered with PES hollow fibre membranes of 300 kDa; then, the permeate was concentrated by a PES hollow fibre membranes with MWCO of 3 kDa (Fig. 3.5). Both UF retentates were first concentrated four-fold and then diafiltered with three volumes of RO water in order to remove smaller proteins and non-protein molecules (sugars, ash, etc.). When protein extraction was preceded by pigment removal, alkaline pH conditions were required to obtain a complete release of the proteins. Protein distribution in both UF retentates was not affected by extraction pH and ethanol pre-treatment. In average, 78–80% of the protein was retained by the 300 kDa membrane. The protein fractions obtained from the UF process had a comparable amino acid profile and distribution of essential amino acids, with the exception of 3 kDa concentrate of pH 7 control, which presented lower amounts of histidine, lysine and phenylalanine.

A flat-sheet membrane of RC with a MWCO of 1 kDa was used to concentrate proteins from native and neutral pH supernatants (5.7 and 7, respectively) obtained after extraction of a water-soluble matrix from *Haematococcus pluvialis* through high-pressure cell disruption (Ba et al. 2016). Both supernatants were ultrafiltered at a TMP of 3.3 bar and a temperature of about 23 °C up to a VRF of 10. The size exclusion chromatography profile of proteins extracted under native pH showed a broad distribution of molecular masses from 584 down to 17 kDa. The presence of a cluster with a molecular weight higher than 600 kDa, composed of proteins, chlorophyll (661 nm) and carotenoids (470 nm) was detected at neutral pH. Low molecular weight proteins (<10 kDa) were detected in the permeate, while both retentates exhibited a protein profile similar to the initial supernatant. These results

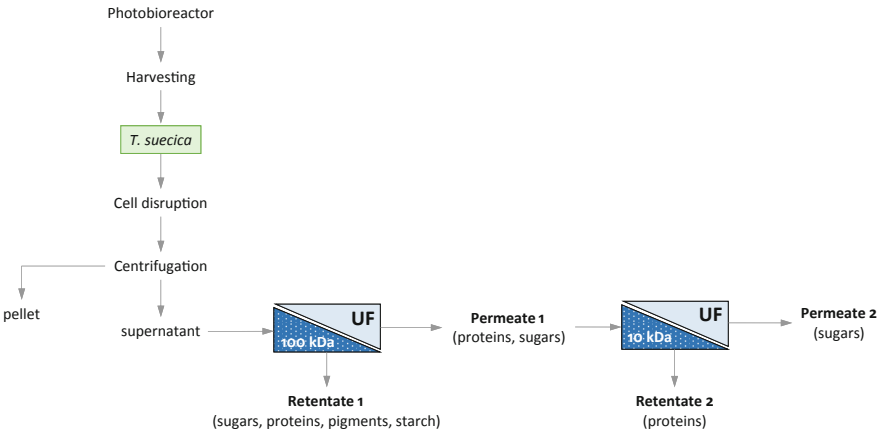

Fig. 3.6 A two-stage ultrafiltration process for separating multiple components of Tetraselmis suecica (UF, ultrafiltration) (adapted from Safi et al. 2014)

indicated the suitability of UF for the concentration of proteins from *Haematococcus pluvialis* with the advantage of using the concentrated solution directly to the recipe for food formulation.

A two-stage UF process for the recovery of microalgae components from the aqueous phase of *Tetraselmis suecica* after high-pressure homogenization was investigated by Safi et al. (2014). The aqueous extract recovered after centrifugation was fractionated with two consecutive PES membranes with MWCO of 100 kDa and 10 kDa, respectively. Starch was completely retained in the first UF process while proteins were recovered in the permeate stream according to their molecular weight (in the range of 15–50 kDa) (Schwenzfeier et al. 2011). The UF membrane allowed also sugars to pass into the permeate. Proteins were retained by the 10 kDa membrane while 65% of total sugars present in the supernatant were recovered in the permeate of the process (Fig. 3.6).

A similar biorefinery approach to obtain an enriched fraction of water-soluble proteins free from chlorophyll from the microalga *Nannochloropsis gaditana* was also investigated by Safi et al. (2017). Cell disruption by high-pressure homogenization produced a greater release of proteins (49%) in the aqueous phase in comparison with the enzymatic treatment with alcalase (35%). The UF process, performed on the supernatant obtained from both cell disruption methods, was studied by using membranes with MWCO of 300, 500 and 1000 kDa. A diafiltration step was also implemented to recover more proteins in the permeate (Fig. 3.7).

The combination of the enzymatic method with UF resulted in a larger overall yield of water-soluble proteins (24.8%) in the permeate compared to the combination of high-pressure homogenization with UF (17.4%). The higher protein yield was attributed to the smaller size of proteins in samples enzymatically treated which simplifies their passage through the UF membranes leading also to higher permeate fluxes in comparison with samples pre-treated by high-pressure homogenization

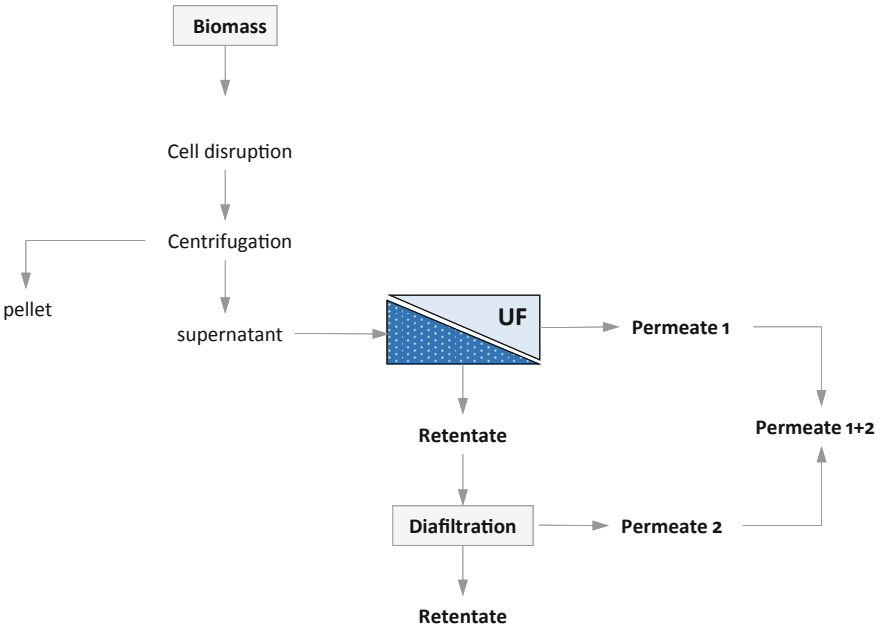

Fig. 3.7 Schematic representation for the recovery of water-soluble proteins from the microalgae Nannochloropsis gaditana (UF, ultrafiltration) (adapted from Safi et al. 2017)

and, consequently, in a better control of the fouling/polarization phenomena. Among the investigated membranes, the highest permeate flux obtained was observed for the 300 kDa membrane for both cell disruption methods. The performance of the UF membranes was negatively affected when membranes with larger cut-off were adopted. This behaviour can be attributed to adsorptive fouling due to retained molecules which can penetrate into the membrane pores of 1000 kDa membranes while remaining only on membrane surface of 300 kDa ones.

Among microalgae, *Arthrospira platensis* (Spirulina), a blue-green coil shaped cyanobacterium, has received more and more attention as a 'superfood' due to its high content of proteins, vitamins, minerals and many essential amino acids and fatty acids (Grosshagauer et al. 2020). In particular, it is an important source of C-phycocyanin (CPC) and allophycocyanin (APC) water-soluble proteins belonging to the phycobiliprotein family (Moraes and Kalil 2009). CPC is a natural blue colourant with an estimated molecular weight of 100–200,000 Da (with α and β subunits of about 20,000 daltons) having a great potential for industrial and commercial exploitation due to its therapeutic properties including antioxidant, anti-inflammatory and anti-cancer activities (Eriksen 2008). Its market value is estimated to be around 10–50 million US$ per annum (Spolaore et al. 2006).

The purification of CPC from Spirulina generally involves a combination of different techniques including sonication, extraction, centrifugation, chromatography, ion exchange and dialysis. The use of membrane processes, and in particular of

barometric membranes, has been also explored for purification and concentration aims thanks to the mild operating conditions which reduce possible denaturation and deactivation of the molecule.

UF membranes with a MWCO of 50 kDa were used by Herrera et al. (1989) to concentrate a Spirulina extract up to a VRF of 1.9. A food grade phycocyanin powder with a purity ratio of 0.74 was obtained after adsorption on activated charcoal and spray drying.

A combination of an aqueous two-phase systems, UF and precipitation was developed by Rito-Palomares et al. (2001) in order to reduce the number of unit operations and increased the yield of the protein. In this approach the use of a 30 kDa UF membrane followed by precipitation with ammonium sulphate led to a protein purity of 3.8 ± 0.1% and an overall product yield of 29.5% (w/w).

Jaouen et al. (1999) investigated the use of MF and UF tubular inorganic membranes for the clarification of raw extracts after sonication of a Spirulina culture while UF, NF and RO tubular organic membranes were investigated for the concentration of the clarified extract. Permeation fluxes in the clarification step were in the range of 35–50 L/m^{2}h (slightly higher for the MF membrane). The retention rate for CPC was of 96% from the beginning to the end of the UF process while its retention increased from 65% to 85% during the MF process. NF membranes exhibited the best performance in the concentration of the clarified extract: in selected conditions of operating pressure and tangential velocity (30 bar and 1.5 m/s, respectively) the recovery of CPC and permeation flux resulted of 100% and 85 L/m^{2}h, respectively.

PAN nanofibre membranes prepared by electrospinning technique and aminated to improve their adsorption capacity for protein molecules showed high binding capacity for both CPC and APC of *Spirulina platensis*; on the other hand, a low selectivity was observed for contaminating proteins which were completely removed during the flow and wash procedures (Liu et al. 2020). The binding force between the APC/CPC and aminated membranes was attributed to multi-type relational interactions between APC/CPC molecules and membranes, including ionic and hydrophobic interactions. The immobilized APC/CPC membranes were considered of great interest as antibacterial and antioxidant materials for water treatment applications.

MF and UF membranes were tested by Chaiklahan et al. (2011) for the purification of a crude phycocyanin extract. The highest purity ratio of crude extract was observed when fresh biomass, rather than freeze-dried, sun-dried and oven-dried biomass, was used as raw material. PP and PES MF membranes with pore size of 5 μm and 0.8/0.2 μm, respectively, were considered suitable for the separation and clarification of crude phycocyanin extract on the basis of phycocyanin recovery (88.6% and 82.9%, respectively) and permeate flux (58.5 and 336 L/m^{2}h). PES UF membranes with MWCO of 50 kDa produced the best results in terms of permeate flux (26.8 L/m^{2}h at 0.69 bar of pressure and 75 mL/min of feed flow rate) and retention rate (99%) in comparison with 70 and 100 kDa membranes.

Recently, Balti et al. (2021) investigated the potential of membrane diafiltration for the fractionation of water-soluble proteins of the supernatant obtained after Spirulina bead milling disruption and centrifugation. The process was investigated

by using tubular inorganic membranes with different MWCO (50, 150 and 300 kDa) or pore size diameter (0.2 μm) with selected operating conditions of pressure (4 bar), cross-flow velocity (5 m/s) and temperature (20 °C).Among the selected membranes, the filtration with the MF membrane allowed a good separation of APC and CPC with more than 89% recovery of APC in the retentate and the production of a blue colour permeate enriched in CPC and free from chlorophyll. Concentrated solutions with more than 80% (dry weight basis) of proteins were obtained.

Hollow fibre UF membranes were used to purify anti-inflammatory peptides from an enzymatic hydrolysate of *Spirulina maxima* obtained by using gastrointestinal endopeptidases (Vo et al. 2013). The hydrolysate was fractionated through membranes of decreasing MWCO (10, 5 and 3 kDa). The <3 kDa fraction contained the highest anti-inflammatory activity and was further purified by anion exchange, gel filtration and RP-HPLC. The end result was the isolation of two peptides consisting of 6 amino acid residues with molecular weight of 655 and 68 Da, which exhibited anti-inflammatory activity.

Membrane filtration has been also investigated for harvesting the Spirulina sp. biomass from the growth medium as alternative methods to coagulation/flocculation and centrifugation. Rossi et al. (2004) evaluated the performance of different polymeric MF and UF membranes (all from Rhodia-Orelis, Miribel, France) in terms of permeation flux and fouling phenomena in the separation of Spirulina from their culture medium. Among the investigated membranes a 40 kDa PAN membrane (IRIS 3038) exhibited the best productivity (steady-state fluxes of about 55 L/m^2h after a 2 h experiment with micro-organisms) and cleaning efficiency. Therefore, it was selected to perform Spirulina harvesting experiments up to a VRF of 10.

The authors evaluated also limiting and critical flux of tubular inorganic UF membranes of 50 kDa (Céram-Inside from Tami, Nyons, France) with fresh biomass, stressed biomass and a suspension of Spirulina enriched in exopolysaccharides (EPS) (Rossi et al. 2008). Permeation fluxes with disrupted cells were similar to those with fresh suspension: although fragments and other released substances from disrupted cells induced additional fouling, the sub-critical zone was not modified. The EPS adsorption appeared as the major fouling phenomenon through the formation of a gel layer which if compressed generates an additive irreversible resistance.

Kanchanatip et al. (2016) characterized the fouling of a submerged membrane bioreactor (MBR) equipped with two UF disc membranes (with PVDF active thin film layer) used to harvest *Arthrospira maxima* cells. Experimental results clearly indicated that fouling is primarily caused by fragmented cells rather than soluble or extracellular polymeric substances. Permeate flux was enhanced by applying membranes with greater pore density and the permeate flux decline was recovered by backwashing the membrane at a pertinent interval.

Recently, Ismail et al. (2021) evaluated the filtration performance of MF and UF membranes in a tilted panel system at different tilting angles for harvesting Spirulina sp. The investigated system was effective and energy-efficient for fouling control. The permeability reached maximum at the tilting angle of 45° thanks to the combination of aeration and panel tilting. Polyvinylidene fluoride (PVDF) MF membranes with pore size of 0.42 μm performed better than PS UF membranes with pore size of

0.04 μm due to the effective impact of air bubbles for foulant scouring that maximized the membrane intrinsic property. High permeabilities (of the order of 540 L/m^{2}h.bar) were achieved under a low energy input of 0.2 kWh/m^3.

3.5 Recovery of Proteins from Agro-Food by-Products

The recovery of proteins from agro-industrial wastes, residues, by-products and wastewaters is a topic of growing interest in the modern agroindustry in agreement with the new concept and development model of circular economy (Castro-Muñoz et al. 2018).

3.5.1 Recovery of Proteins from Cereal by-Products

Rice by-products, generated through the milling processes, are recognized as a potential source of bioactive compounds, such as proteins, essential amino acids and phenolics (Zaky et al. 2020). Bran and broken rice are the two main rice co-products. Broken rice, containing about 80% starch and 8% proteins, is generally used by the starch industry to extract powder and crystal starch obtaining proteins as the main by-product (Shih 2012).

The recovery of proteins from hydrolysed rice bran by UF was investigated by Hamada (2000). Protein hydrolysates obtained after centrifugation of defatted rice bran treated with alcalase were ultrafiltered by using two different spiral-wound membranes with MWCO of 1 and 3 kDa. 3 kDa membranes removed all small peptides of less than 3 kDa from all hydrolysates; however, a substantial loss of protein (up to 35%) due to their permeation was observed. On the other hand, 1 kDa membranes increased protein yields despite the increased membrane area and operating time. Membranes with MWCO of 2 kDa were considered more suitable to reach a higher purification degree due to the removal of phytic acid and its degradation products, including inositol penta- and tetraphosphates.

Ferri et al. (2017) optimized an environment-friendly process for the hydrolysis of protein by-products derived from the rice starch industry. Commercial proteases were tested to hydrolyse the by-product, without any initial pre-treatment. Among them, protamex, alcalase and neutrase produced peptide fractions with valuable bioactivities without resulting cytotoxic or irritant. After centrifugation the digestate was fractionated by using PES flat-sheet membranes in order to isolate peptide samples with different molecular weights. Suspended solids and undigested proteins and peptides were removed in the first step by using an MF membrane with a pore size of 0.2 μm. UF membranes with 8, 5 and 1 kDa were then used in sequential design to treat the MF permeate. The highest peptide content was detected in the retentate of the 8 kDa UF membrane and the antioxidant activity of all fractions was in agreement with the protein content. Samples treated with protamex exhibited the highest antioxidant activity (between 1.4 and 5.1 gAA/L in the UF retentates).

Ground sweet sorghum grain can be fermented to ethanol and the fermentation residue (stillage) after distillation can be separated into distillers' grain (by filtration), centrifuge solids and stillage solubles (by centrifugation). A combination of UF and RO membranes was investigated by Wu (1987) in order to produce a concentrated protein fraction with potential feed use from the stillage solubles. CA and PS membranes with a MWCO 10 kDa were selected in order to remove large molecules before the RO process. At an operating pressure of 6.8 bar the CA membrane exhibited higher permeate flux and smaller amounts of nitrogen and solids in the permeate stream than the PS membrane. In particular, the permeate of the PS membrane accounted for 43% of nitrogen, 55% of solids and 72% of the ash of stillage solubles. For the CA membranes values were of 36%, 44% and 71%, respectively. The UF permeate of PS and CA membranes was processed with a spiral-wound RO membrane in polyamide (SW30–2521, Filmtec). Permeate streams produced at VRF of 4.2 were considered suitable for reuse or disposal while the small volume of concentrate was considered of interest for food application.

The use of the UF process within a traditional corn ethanol process to recover proteins before the fermentation step was investigated by Leberknight et al. (2011). Among the investigated membranes (5 kDa and 100 kDa RC, and 5 kDa and 100 kDa PES membranes) the 5 kDa RC membrane provided the best results due to high yield and sustained resistance to fouling. Cake formation was the major resistance with RC membranes while irreversible pore blockage and constriction were dominant with PES-based membranes. Ash, protein, sugars and oils were all identified as fouling components. The integrated process of extraction, UF, fermentation and distillation from corn kernels is depicted in Fig. 3.8.

UF membranes with MWCO of 5 and 30 kDa retained more than 92% of proteins from brewer's spent grain (Tang et al. 2009). The protein content in the final product was of about 20% and 16%, respectively. It resulted much higher in comparison with that obtained by rotary evaporation (4.86%).

3.5.2 Recovery of Proteins from Soy Processing Wastes

Wastewaters of soybean manufacture contains valuable compounds such as proteins and sugars; their recovery appears of great interest in order to reduce the polluting load of produced effluents and simultaneously utilize valuable resources.

The separation of soluble protein from soy processing waste liquors can be accomplished by UF. Moure et al. (2006) evaluated the use of three different polyethersulphone (PES) membranes (with MWCO of 10, 30 and 50 kDa) in flat-sheet configuration in the treatment of waste liquors generated in a plant making protein concentrates from defatted soybean meal by acidic treatments. Operating at selected transmembrane pressures, protein rejections were of 70.5%, 74.7% and 63.7% for the 10, 30 and 50 kDa membranes, respectively. The enzymatic hydrolysis of fractions of higher molecular weight (30–50 and > 50 kDa) allowed to obtain products with improved emulsifying activity and stability, particularly for hydrolysates with a degree of hydrolysis between 20% and 30% (Moure et al.

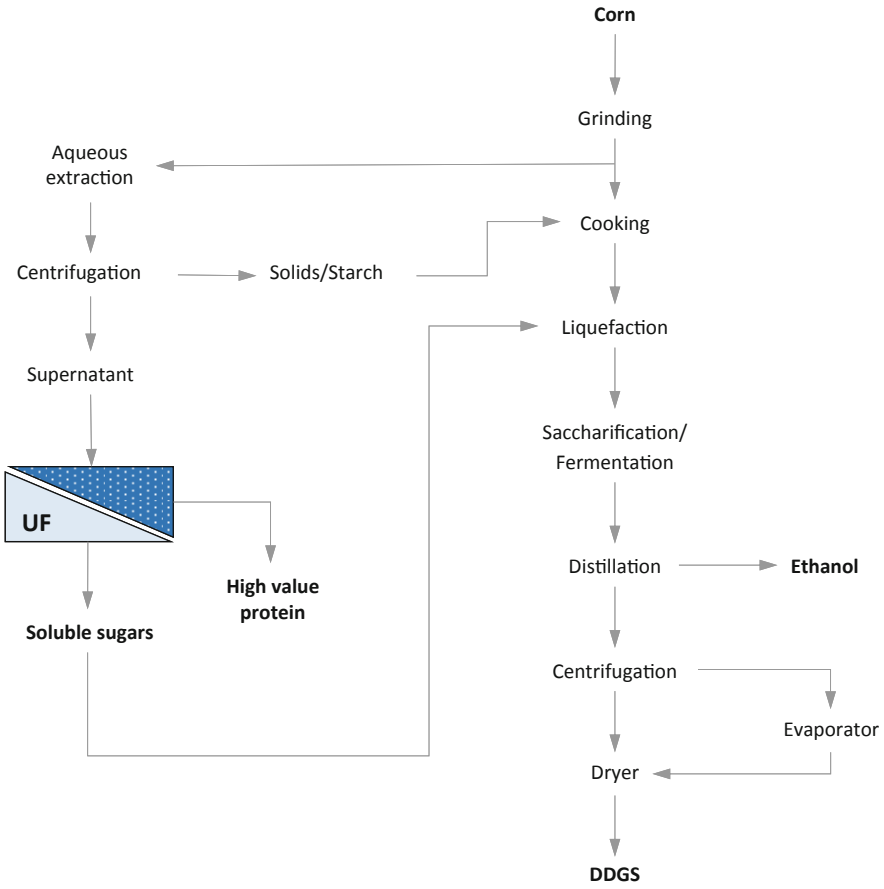

Fig. 3.8 Integrated process for the production of ethanol and recovery of proteins from corn kernels (UF, ultrafiltration; DDGS, dry distiller's grain with solubles) (adapted from Leberknight et al. 2011)

2005). Hydrolysates from the fraction with molecular weight between 30 and 50 kDa showed the highest hydroxyl radical-scavenging capacity, while hydrolysates from the fraction with molecular weight higher than 50 kDa showed the highest Trolox Equivalent Antioxidant Capacity (TEAC) (Moure et al. 2008).

Cassini et al. (2011) analysed the fouling tendency of UF tubular membranes of different MWCO (5, 20 and 50 kDa) in the treatment of SPI wastewater. The fouling tendency increased by increasing the MWCO from 65% (for the 5 kDa membrane) to 76% (for the 50 kDa membrane). According to Hermia's Model (Hérmia 1982) the complete pore blocking was the predominant fouling mechanism for all the UF membranes investigated.

A membrane-based process for extracting soy protein, oligosaccharide and isoflavone from soybean wastewater was patented by Jiang and Wang (2013). In this

approach, UF membranes retain soybean proteins and the concentrated solution can be spray-dried to obtain a pure soybean protein powder. Soy isoflavones in the UF permeate are adsorbed on weak polar macroporous resins and then eluted with ethanol. Soybean oligosaccharides in the resin effluent liquid can be concentrated by NF membranes and the concentrated liquid can be used for preparing oligosaccharide powders. The final treatment of the NF permeate by RO produces pure water through the removal of inorganic salts.

Recently, the flocculation with chitosan followed by UF with a 5 kDa RC membrane has been studied by Cheng et al. (2017) to remove proteins from soybean whey wastewater. Under optimized conditions of flocculation (dosage of chitosan 0.8 g/L, pH 5.5, temperature 30 °C, 60 min) maximum protein removal of 61.21% was achieved.

3.5.3 Recovery of Proteins from Pea Whey Discharge

The production of pea protein concentrates and isolates by wet fractionation generates a large amount of effluent. The major stream of discharge is the pea whey, a liquid fraction containing a large portion (20–30 g/100 g) of pea protein obtained from the centrifugal separation of upstream protein slurry. The pea whey discharge not only results in protein loss, but also creates serious environmental problems.

UF membranes represent a useful approach for the recovery of proteins from these effluents (Vose 1980). Gao et al. (2001) investigated the use of UF membranes of 10 and 30 kDa in spiral-wound and hollow fibre configuration, respectively, for the recovery of proteins from the commercial pea whey discharge according to the schematic diagram depicted in Fig. 3.9. The pea whey was previously centrifuged (10,000 × g) to reduce solids load or bag-filtered (25 μM) to remove abrasive particulates. The UF treatment with the 10 kDa membrane followed by diafiltration increased the protein content from 39 g/100 g solids in the pea whey discharge to 84 g/100 g solids in the retentate. On the other hand, the protein content of the retentate obtained with the 30 kDa membrane was of 37.5 g/100 g. Therefore, this membrane was ineffective in concentrating and purifying pea whey proteins. Preliminary results indicated that protein isolates from the UF process have superior functional properties and nutritive value to the conventional commercial product.

3.6 Conclusions and Future Trends

Barometric membrane processes represent a viable approach for the recovery of protein-based compounds from natural sources and agro-food by-products. In addition, the combination of proteases and membrane fractionation of peptides is an interesting tool to improve the potential of proteins with high nutritive value but poor functional and sensorial properties.

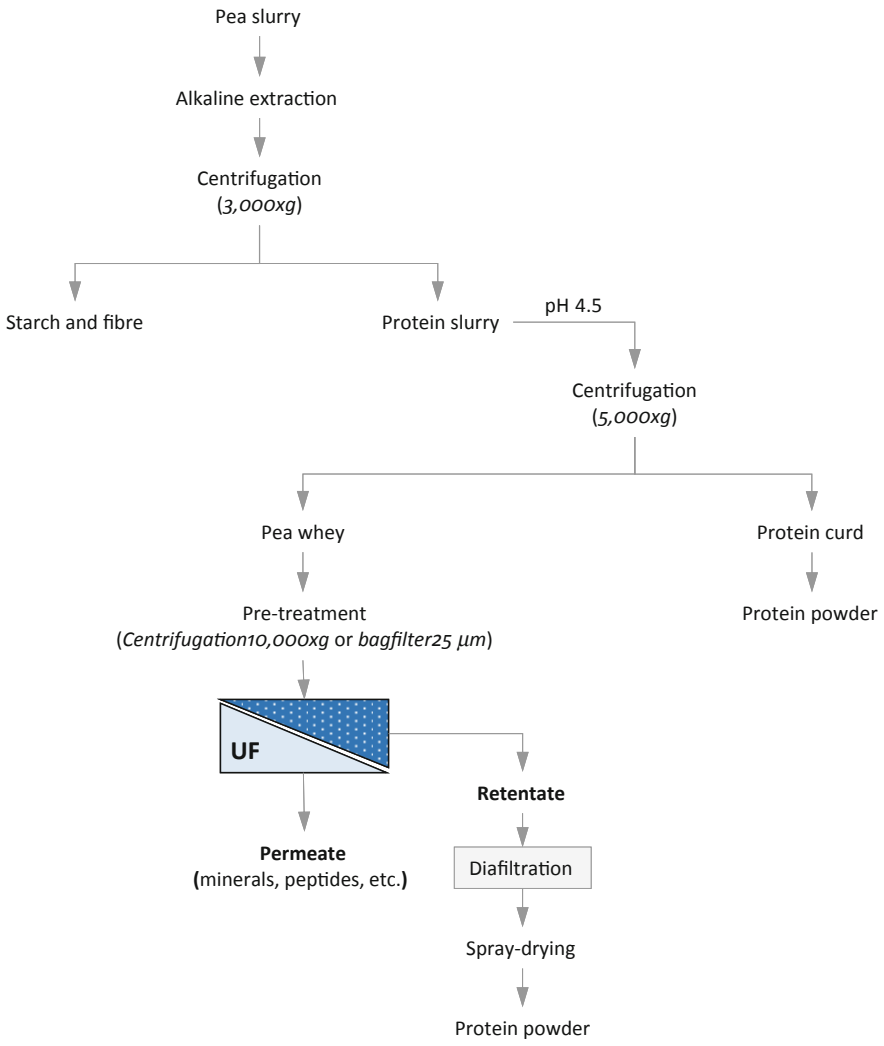

Fig. 3.9 Schematic diagram for the recovery of proteins in the effluent discharge (pea whey) of a wet milling process (adapted from Gao et al. 2001)

Typical membrane-based applications concerning the production of soy protein isolates and concentrates as well as the fractionation of protein hydrolysates from different sources including mushrooms, microalgae and cereals processing wastewaters have been presented and discussed in this chapter.

Further investigations are needed in order to guarantee the success of these processes at the industrial level, particularly regarding the reduction and control of membrane fouling. At this purpose the development of new membrane materials as well as the modification of those already existing offers interesting opportunities to

improve membrane productivity and to extend membranes' lifetime. In addition, the combination of membrane unit operations among them or with emerging extraction technologies will offer a great potential to optimize the recovery and the purification of protein-based compounds from the original sources or from agro-food by-products in agreement with circular economy and process intensification strategies.

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Electro-Activation as Emerging Technology for Proteins Extraction from Plant Materials: Theory and Applications

4

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Abstract

Electro-activation of aqueous solutions is a branch of the applied electrochemistry. It consists in modifying the reactivity of solutions following adequate treatment with an electric field. In this chapter, fundamentals of the electro-activation technology are highlighted. The main electrochemical reactions occurring at the anode-solution and cathode-solution interfaces are discussed. The possibility of exploiting these reactions to produce aqueous solutions having acidic or alkaline characteristics is highlighted and the possibility of using them as extracting agents is justified. Moreover, the present analysis of the fundamentals of electro-activation technology reveals its high potential to be used as an eco-friendly technology because of the unique possibilities it offers to use electro-activated solutions instead of chemical alkaline solutions which need special handling conditions. Finally, some practical uses of electro-activated solutions for the extraction of proteins and protein-rich extracts from plant materials are discussed. Electro-activated solutions having alkaline properties were successfully used for proteins and protein-rich extracts from canola and soybean meals. The plant extracts obtained by using electro-activated solutions showed the high potential of the electro-activation technology for proteins extraction from plant-based materials such as canola (rapeseed), soybean meals. The use of the electro-activation technology for plant proteins extraction can also be extended to other plant materials recognized as high source of valuable proteins such as different pulses and grains.

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Plant proteins · Extraction · Electro-activation · Efficiency · Functionality · Sustainability

4.1 Introduction

The world is continuously facing huge challenges which are of great importance to the humanity. Among the major challenges that humans have to face, the question of food security is one of the most important. In fact, an increase in the global demand for proteins is witnessing. This is because the sources of animal proteins are very limited, in addition to the problems that their production generates, particularly those related to greenhouse gases and ethical considerations such as the use of very large land surfaces for crops production that are used in animal feeding instead of human nutrition. In this context, the alternative is to use more sustainable protein sources and for that, plant-based proteins have the overall scientific and social consensus that it is the preferred solution (Day 2013; Sá et al. 2020).

Plant proteins can be obtained by extraction from different sources such as pulses (pea, chickpea, and lentil) and different beans such as soybean and bean since they are characterized by high protein content ranging between 20% and 35% on weight basis. They can also be obtained from different residual meals of oilseeds such as soybean, rapeseed, flaxseed, and sunflower. Indeed, the different residual meals remaining after oil extraction usually contain high crude protein level which varies from 25% to 45% on dry basis. Moreover, high quality proteins can be also obtained from other cultures such as oat, barley, wheat, millet, amaranth, and sorghum (Kumar et al. 2021; Freitas et al. 2021; Kamal et al. 2021).

Usually, plant proteins are extracted in alkaline media by using different alkalinizing reagents such as sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), as well as quaternary ammonium compounds. In industry, NaOH is the most used chemical for plant proteins extraction due to its easy availability and effectiveness for pH adjustment (Braspaiboon et al. 2020). To enhance the extraction efficiency, high temperatures are also used in combination with optimized parameters such as the meal/solution ratio, mechanical agitation and particle size reducing by grinding. Moreover, the efficacy of these parameters is time dependent. After that, the treated slurry is centrifuged or filtered by passing through a filter-press and the supernatant is recovered for further treatment by using acidifying agents such as hydrochloric acid (HCl) or sulfuric acid (H_2SO_4) for isoelectric precipitation of the extracted proteins in the aqueous phase. After acid precipitation, the proteins are recovered by centrifugation, generally neutralized and spray-dried (Aider and Barbana 2011; Momen et al. 2021). It can be deducted from this simplified process diagram of plant proteins production from different flours and meals that huge quantities of alkaline and acid wastes are generated. It requires special equipment and facilities for sewage treatment because their release into the environment is extremely pollutant since they contain residual

organic and inorganic matters with different chemical and biological oxygen demands (Khedkar and Singh 2018; Westgate and Park 2010). Furthermore, the use of alkali and acids at industrial level requires that they must be handled under strongly controlled conditions because of their hazardous character since they are delivered in a highly concentrated form.

To overcome the aforementioned disadvantages of using chemical reagents in the process of plant proteins extraction, it is possible to use the electro-activation technology which can be considered as a reagentless and highly promising technology for sustainable use in the food industry.

The aim of the present chapter is to highlight the principles of aqueous solutions electro-activation, the use of the electro-activated solutions as replacement of acids and alkali in the process of plant proteins extraction, and to establish some future orientations of using electro-activation technology as a sustainable approach in the food industry.

4.2 Theory of Electro-Activation

4.2.1 Definition and General Equations

Electro-activation of aqueous solutions is a branch of applied electrochemistry which is based on the phenomena of water electrolysis and the oxidation–reduction reaction occurring at the electrodes–solutions interfaces under controlled operating condition with an appropriate designed electro-activation reactor (Aider et al. 2012; Gerliani et al. 2019c; Gerzhova et al. 2015b). It consists of exciting the solution by applying an external electric field in a reactor that is modulated by appropriate disposition of anionic and cationic exchange membranes in order to control, to some extent, the flow of charged ionic species, and to provide the activation energy necessary to achieve targeted chemical reactions (Aider et al. 2012). Electro-activation of aqueous solutions is a heterogeneous process involving ion exchange and excitation of charged particles to higher energetic level which is not possible to achieve under a free-flowing conditions. Indeed, electrons inside atoms can be excited by local electric fields of high intensity. The electric fields can be strong enough to induce ionization of the atom, the free electrons then become a source for further ionization processes and reactivity. In this context, the importance of appropriate disposition of ion exchange membranes is a key factor (Aider et al. 2012).

The electro-activation reaction velocity (kinetics) can be described by the following equation (Eq. 4.1) (Korko 2013):

$$\mu = k \cdot A_i \cdot \Delta C \cdot e^{\left(-\frac{E_a}{R \cdot T}\right)} \tag{4.1}$$

μ: Reaction velocity, mole s^{-1} m^{-3}
k: Mass transfer coefficient, m s^{-1}

Ai: Specific reaction surface area, $m^2\ m^{-3}$
ΔC: Concentration (activity) gradient at the membrane–solution interface of a targeted ionic specie, mole m^{-3}
Ea: Activation energy, J $mole^{-1}$
R: Universal gas constant, J $mole^{-1}\ K^{-1}$
T: Temperature, K

It is also known that the overall mass transfer coefficient (k) in heterogeneous chemical and electrochemical processes is dependent of the reaction rate constant (k_i) and of the diffusion coefficient (D) of a given ionic species from the bulk solution to the zone of the reaction. So, $k = f(k_i, D)$.

Furthermore, it has been demonstrated that the electrochemical reactions occurring during electro-activation processes generally follow a first-order reaction. Thus, the reaction rate constant (k_i) can be defined according to the Arrhenius equation as follows (Eq. 4.2):

$$k_i = k_0 \cdot e^{\left(-\frac{E_a}{R \cdot T}\right)} \tag{4.2}$$

In Eq. 4.2, k_0 is the rate constant of this given first-order reaction, s^{-1}. It can be experimentally calculated.

If an ion exchange membrane is placed between the anode and cathode, the variation of an ionic species concentration (mole m^{-3}) can be calculated by using the Faraday's law as follows (Eq. 4.3) (Sundén 2019):

$$\Delta C_{i,j} = \eta_{i,j} \cdot \frac{I \cdot \tau}{F \cdot V_s} \tag{4.3}$$

$\eta_{i,j}$: Electric current efficiency (% or ratio), which is defined as useful power output divided by the total electrical power consumed. It represents the ratio of the electrochemical equivalent current density for a specific reaction to the total applied electric current density. It describes the efficiency with which charge (electrons) is transferred in a system facilitating an electrochemical reaction.
$_{i,j}$: Ionic species $_i$ relatively to an output variable $_j$.
I: Nominal electric current intensity between the anode and cathode, A.
τ: Reaction time, s,
F: Faraday constant, C $mole^{-1}$.
Vs: Solution volume in the anodic or cathodic compartment of the reactor, m^3.

Thus, under the effect of the applied external electric current, charged soluble species can be transported through an ion exchange membrane to the corresponding electrode or repulsed by the membrane if the electric charge is of a repulsive type. This is a general concept which applies to ionic species electro-migration. Thus, the controlled modification of given ionic species at each side of the ion exchange membrane can be used in different technological processes such as concentration

of a targeted element or to enhance its availability in given aqueous solution by creating high concentration of the targeted ionic species in order to enhance its participation in a desired chemical reaction. Furthermore, it is also possible to separate electrolytes from non-charged molecules.

4.2.2 Role of Water in the Electro-Activation Process

Water is very important in the formation of the charged and highly excited ionic species during the electro-activation process. However, from a process engineering point of view, water by its nature is not an electric current carrier and the presence of electrolytes in the medium is a requirement to initiate the electrolysis process of water. The minimal required electrolyte initial concentration can be as low as few ppm. Furthermore, the type of electrolyte is also very important to achieve the targeted oxidation–reduction reactions because of their different affinities to the electrode materials. In the case where the objective is to obtain acidic and alkaline solutions at the anode and cathode compartments, respectively, the overall reactions of water electrolysis, responsible for the generation of H^+ and OH^- ions are summarized in Eqs. 4.4 and 4.5, as follows:

$$\text{At the cathode surface } (-): 2H_2O + 2e^- \rightarrow H_2 \uparrow + 2OH^- \quad (4.4)$$

$$\text{At the anode surface } (+): 2H_2O - 4e^- \rightarrow O_2 \uparrow + 4H^+ \quad (4.5)$$

Indeed, some minerals can undergo the oxidation–reduction process whereas other minerals cannot compete with water for the electrode attraction. Thus, in the case where elements such as Li^+, K^+, Ba^{2+}, Ca^{2+}, Na^+, Mg^{2+}, Be^{2+}, and Al^{3+} constitute the electrolyte, water molecules are oxidized and reduced at the corresponding electrodes because the aforementioned metal elements do not take part in the oxidation–reduction reactions. The competition for the electrode is favorable for water molecules, thus H^+ and OH^- ions are easily formed. At the same time, gaseous oxygen and hydrogen are formed in the anodic and cathodic compartments of the electro-activation reactor. Saturation of the catholyte by hydrogen gas allows a formation of highly reducing medium whereas the formation of oxygen and other ionic species with high electronegativity permits a formation of highly oxidizing medium in the anolyte solution. In the situation described by Eqs. 4.4 and 4.5 and in the presence of minerals such as Na^+ or K^+, it is possible to obtain a strong base in the cathodic compartment and a strong acid in the anodic compartment if Cl^- ions constitute the electrolyte of the anodic solution. This can be summarized by the following equations (Eqs. 4.6 and 4.7) as follows:

$$\text{In the cathode compartment } (-): 2H_2O + 2e^- + 2Na^+ \rightarrow H_2 \uparrow + 2NaOH \quad (4.6)$$

$$\text{In the anode compartment } (+): 2H_2O - 4e^- + 4Cl^- \rightarrow O_2 \uparrow + 4\,HCl \quad (4.7)$$

The aforementioned reactions of producing a base and acid are typical of classical electrolysis. To obtain high solution reactivity (high chemical potential) it is necessary to bring the solutions to higher energetic and excitation level. This is possible by means of appropriate disposition of anion and cation exchange membranes between the anodic and cathodic sides of the electro-activation reactor. Under such conditions, excessive accumulation of highly charged ionic species without a possibility of flowing through the membranes to the opposite electrode creates a highly excited ionic species. This condition can be expressed as a metastable state of the solution. Under such conditions, the activation energy of a chemical reaction involving a solution in a metastable state can be significantly reduced compared to the required activation energy under normal conditions. At the same time, it is known that any non-equilibrium interaction accompanied by acceleration or deceleration of the motion of electrons of a substance leads to a relatively stable change in the electronic distribution functions within the atomic and molecular levels. However, non-equilibrium processes, the result of which is the shift of equivalent amounts of electrons toward both external and internal activation barriers, cannot significantly affect the interaction energy of this substance in the process of subsequent chemical reactions. Only processes directed by a displacement of the distribution function for the electrical and (or) magnetic components of energy can be practically used to regulate the rate and direction of the development of the chemical interaction of substances. The transformation of low energies that remain in a substance after the cessation of the activating effect is largely explained by the kinetic parameters of the process of interaction of atoms, molecules, and ions.

By using the Butler–Volmer equation (Dickinson and Wain 2020), it is possible to calculate the local electric current intensity (i_{loc}) by the following equation (Eq. 4.8):

$$i_{loc} = i_0 \left[\exp\left((1-\alpha) \cdot \frac{F \cdot \eta}{R \cdot T} \right) - \exp\left(-\alpha \cdot \frac{F \cdot \eta}{R \cdot T} \right) \right] \tag{4.8}$$

In (Eq. 4.8), i_0 is the equilibrium current, called the exchange current density. By definition $i_0 = i_{co} = i_{ao}$, where i_{c0} is the equilibrium cathode current and i_{a0} is the equilibrium anode current. F is the Faraday constant, η is the overpotential, R is the gas constant, T is the thermodynamic temperature, and α is a quantity called the transfer coefficient (%).

This expression shows the exponential dependence of the current on the electrode potential with respect to the equilibrium value. So, the shift from this equilibrium state is called over-voltage or polarization. It has little value for reversible processes and a significant value for irreversible ones. The higher the exchange current, the higher the actual current at a given over-voltage. The exchange current is related to the reaction rate constant and to the concentration of ionic species in the solution. Analysis of this equation reveals that by using appropriate reactor configuration, namely the disposition of electrodes, anion and cation exchange membranes, locally the current intensity can reach values that are thousands of times greater than the nominal (rated) current measured between the anode and cathode. Such a situation

can excite the electrons on the charged particles and bring them to much higher energetic level, making them by the way more reactive. This situation occurs in the electro-activation process and is responsible of the higher reactivity of electro-activated aqueous solutions compared to their chemically equivalent ones. This situation is possible only when high nominal current intensities are used (Okajima et al. 2010).

At low over-voltages, the linearized Butler–Volmer expression is used and can be written as follows (Eq. 4.9):

$$i_{loc} = i_0 \left[(\alpha_a + \alpha_c) \frac{F}{RT} \cdot \eta \right] \tag{4.9}$$

where α_a and α_c are the anodic and cathodic transfer coefficients, respectively.

The current passing through the surface of the electrode–electrolyte has only one direction. Then, at significant over-voltages, one of the two terms (α_a or α_c) in Eq. 4.9 can be neglected. At low values of over-voltage, the current equation is simplified as follows (Eq. 4.10):

$$i_{loc} = i_0 \left[\frac{F}{RT} \cdot \eta \right] \tag{4.10}$$

Kinetic parameters such as exchange current and transfer coefficient can be determined through the logarithmic form of the Tafel equation (Eq. 4.11) (Gileadi and Kirowa-Eisner 2005; Petrii et al. 2007).

$$\eta = \pm A \cdot Log_{10} \left(\frac{i}{i_0} \right) \tag{4.11}$$

where η is the overpotential, A is the Tofel slope (V), i is the current density ($A.m^{-2}$), and i_0 is the exchange current density ($A\ m^{-2}$).

The transfer coefficient affects the symmetry of the current intensity as a function of the current voltage curve with respect to the equilibrium potential which is symmetric if $\alpha = 0.5$, a larger value for reduction processes, a smaller value for oxidative ones. Thus, for example, when NaCl is used as electrolyte and depending on the used electric current intensity, the following ionic and chemical species can be formed at the anode-solution interface (Eq. 4.12) (Bessarabov and Millet 2018):

$$\left.\begin{aligned} &2H_2O - 4e^- \rightarrow 4H^+ + O_2 \\ &2Cl^- - 2e^- \rightarrow Cl_2 \\ &Cl_2 + H_2O \rightarrow HClO + HCl \\ &HClO \leftrightarrow H^+ + ClO^- \\ &2ClO_2 + O_3 + H_2O \rightarrow 2ClO_3^- + O_2 + 2H^+ \\ &HCl + 2H_2O - 5e^- \leftrightarrow 5H^+ + ClO_2 \end{aligned}\right\} \tag{4.12}$$

At the cathode-solution interface, hydrogen is generated as gas and metals are reduced (Bhardwaj and Balasubramaniam 2008). In the cathode chamber, reactions

take place to form hydroxides and carbonates, for examples. The hydroxides are used as alkaline solutions to achieve different reactions occurring under high pH values (Eq. 4.13) (Coutanceau et al. 2018).

$$\left.\begin{aligned} &2H_2O + 2e^- \rightarrow H_2 + 2OH^- \\ &2H_2O + 2Na^+ + 2e^- \rightarrow H_2 + 2NaOH \\ &2Na^+ + 2HCO_3^- \leftrightarrow Na_2CO_3 + H_2O + CO_2 \end{aligned}\right\} \quad (4.13)$$

The intensity of the electrochemical reactions in an electro-activation reactor is due to the electron conductor which meets the ionic conductor, i.e., at the electrode–electrolyte interface, but also to the role of the membranes that are not allowing some ionic transfer and, thus creating a highly non-equilibrium state at the solution–membrane interface. The particularity of this specific region, considered to be a surface phase, is the existence of a specific structure of particles and the presence of an electric field of extremely high intensity which can reach up to 10×10^6 volts/cm^2 across it. The electric field is caused by the separation of charges that are present between the two bulk phases in contact. In such a situation, the surface phase can be considered as a parallel plate condenser, with one plate on the center of the ions that have been brought to the electrode, at the distance of their closest approach to it, and with the second plate at the metal surface; between the two plates and acting as a dielectric material (i.e., a nonconducting material) are oriented water molecules (Despić and Bockris 2011).

4.3 Conventional Chemical Plant Proteins Extraction

Technological processing of plant proteins extraction can be divided into mechanical, physical, and chemical. Mechanical methods include grinding, sieving fractionation, and air separation, but these methods make it possible to obtain flour and concentrates with a protein content of no more than 50–60%. Physical methods mainly involve heat treatment, the purpose of which is to break down individual substances, for example, specific low molecular weight peptides and enzyme inhibitors. It should be noted that heat treatment (for example, in meal toasting) leads to irreversible denaturation of protein compounds, however, it has a positive effect on the digestibility and nutritional value of the protein (Salazar-Villanea et al. 2016; Mosenthin et al. 2016). Chemical processing of meal or any protein-containing plant material is carried out in order to obtain concentrates and isolates of plant protein for feed and food industries. It is also known that plant materials contain phenolic compounds, such as chlorogenic, quinic, and caffeic acids, among others (Yang et al. 2021). Along with them, phenolic compounds similar to isoferulic and synapic acids, as well as esters of oxycinnamic acid, which cause darkening of products during heat treatment, are found in meals remaining after oil extraction. The negative effect of high concentration of chlorogenic acid is manifested in the inhibition of trypsin and lipase, so its level should not exceed 1% (Shchekoldina and Aider 2014). It should be noted that from scientific literature

data it is known that chlorogenic acid inhibits trypsin and lipase activities (Narita and Inouye 2009). Protein isolates obtained from meal using weak alkali solutions contain neo-chlorogenic and isochlorogenic acids along with the already indicated acids. Under the action of polyphenol oxidase, chlorogenic acid is converted into quinones, which form dark-colored compounds of unknown composition. There are known works in which chlorogenic acid is considered as a regulator of growth and as a protective factor in relation to some microorganisms (Lou et al. 2011). The chemical extraction of proteins in industry includes extraction using alkalis, acids, enzymes, or saline solutions, followed by separation of the extract. Further, the protein is separated from the accompanying components and concentrated. Quite often, the meal is treated with NaOH solution under heating, followed by the precipitation of the protein at the isoelectric point (IEP) from the obtained extract. Then the precipitate is separated, neutralized, and dried. Alkaline extraction can be carried out at pH 9–13 with further clarification and protein precipitation at the IEP by acidification to pH 3.5–5 (Tan et al. 2011). In order to increase the solubility of the target product and its purity, the extract is clarified at pH 7.5–8.5 following a centrifugation treatment to eliminate the insoluble matters. The precipitation of proteins is also carried out with the help of organic solvents such as ethanol, or acetone in some cases, which disrupt the hydrophobic interaction in protein molecules, as well as with the help of concentrated salt solutions, which disrupt the hydration shell of proteins. In the case of salting out, the solution becomes supersaturated due to a lack of solvent, since part of the water goes not to dissolve the protein, but to dissolve the salt (Hyde et al. 2017; Duong-Ly and Gabelli 2014). As a result, protein molecules stick together, forming large particles that precipitate out of solution. Different studies reported developments on the extraction of protein from plant sources by using conventional (solvent and alkali-based) and advanced green extraction technologies such as biochemical extraction assisted with mobilized or free enzymes, physical extraction assisted with ultrasound, pulsed electric field, microwave, and high pressure (Görgüç et al. 2020). All the proposed techniques are aimed to enhance the plant cell disruptive capacity for more efficiency with respect to protein recovery and minimal environmental pollution. However, the huge volumes of different wastes are still a problem in this industry (Kumar et al. 2021).

4.4 Extraction of Plant Proteins by Using the Electro-Activation Technology

The feasibility of extracting plant proteins by using the electro-activation technology is based on the created strong alkaline and acidic conditions in the cathode-solution and anode solution interface, respectively. Indeed, following water electrolysis and adequate membrane modulation of the ionic species electro-migration, it is possible to create extracting conditions which can effectively affect the protein-containing material in order to facilitate the proteins release into the surrounding solution. This is ensured by both the medium pH and the solutions enhanced reactivity following their electro-activation into catholyte (alkaline) and anolyte (acid) (Gerzhova et al.

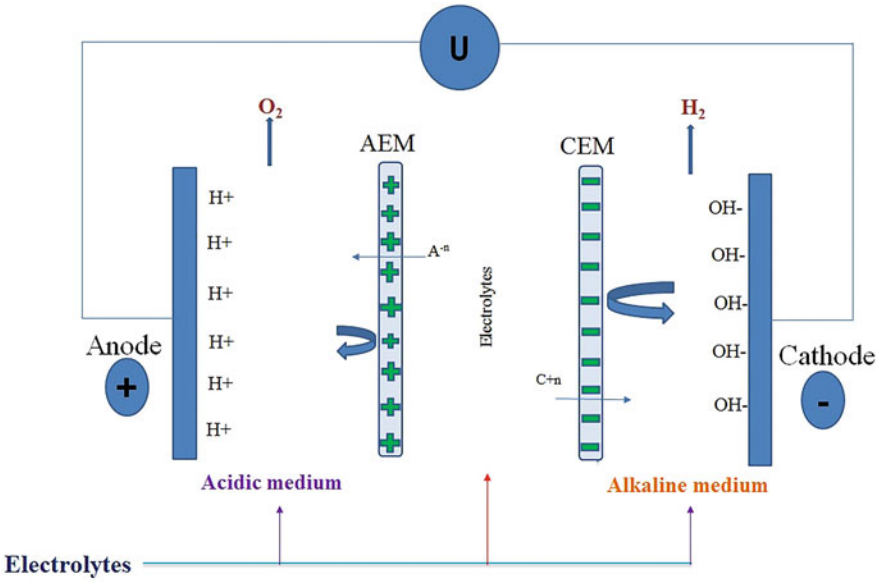

Fig. 4.1 Schematic representation of the main reactions occurring at different sides of the electro-activation reactor. A^{-n} and C^{+n} are anions and cations, respectively. AEM: Anion exchange membrane. *CEM* Cation exchange membrane

2015a; Aider et al. 2012). The solutions (catholyte and anolyte) are obtained in the cathodic and anodic compartments of the electro-activation reactor, as shown in Fig. 4.1.

Electro-activated solutions (alkaline and acidic) can be generated in a batch and continuous mode. The batch operating mode to produce electro-activated solution as well as the continuous mode can be designed by combining different parameters such as the number of the used membranes, the electrode-membrane separating distance, the solutions flow rate, the electric current density, and the duration of the electro-activation treatment. The batch mode is easily used by connecting a receiving tank to the corresponding compartment of the electro-activation reactor to ensure that the solution is enough activated to achieve the targeted properties such as pH and extracting ability. In the continuous mode, the solution is completely electro-activated in situ of the reactor before it is discarded to the receiving tank or directly to the using unit operation. Also, it is important to mention that for both batch and continuous operating mode, several electro-activation units can be connected in a serial mode.

4.4.1 Canola Proteins Extraction

Canola proteins are characterized as very heterogeneous because of the diversity of their molecular weights and isoelectric points. This finding was in agreement with

the study of Quinn and Jones (1976) who reported over 30 protein species with two major proteins; cruciferin being a neutral protein of a high molecular weight (300–310 kDa) and an isoelectric point around pH 7, and napin, which is a small molecular weight protein (12.5–14.5 kDa) characterized by high solubility in the pH range of foods and beverages (pH 3–7) because of its strong alkaline character and its isoelectric point which is situated around pH 10–11 (Quinn and Jones 1976). However, from a quantitative point of view and for the optimization of the extraction yield of proteins from plant materials, precipitation of proteins at pH close to 4.3 is largely used in the industry. Indeed, the minimal solubility was reported to be around pH 4.3 and many plant proteins have their isoelectric points within slightly acid pH region (pH 4-5). Gerzhova et al. (2015a) studied the possibility of using electro-activated alkaline solutions as extracting agents in a process consisting of extraction of canola proteins from canola meal. In this study, it has been reported that the pH has a major effect on the extractability which is in accordance with other publications (Ghodsvali et al. 2005; Klockeman et al. 1997; Nioi et al. 2012). Total dry matter extractability using electro-activated alkaline solutions was compared to the conventional alkaline extraction using NaOH and the reported results did not show any significant difference. Extractability was slightly increased with an increase in NaCl concentration. The effect of current intensity was studied and a minimal amount of extracted dry solids was reported under a current intensity of 200 mA with an average value of 19.67 ± 1.83%. The highest extraction yield of the total dry matter was 32.03 ± 4.97% by using an alkaline electro-activated solution which was produced under 300 mA during 60 min of electro-activation. This solution was characterized by high extracting potency.

The tested parameters impacted the protein extractability comparatively more than the total yield dry solids with the current intensity being the most significant factor. Maximum protein extractability under 200 mA was 19.93 ± 1.39% which was significantly lower in comparison with conventional extraction. With 300 mA, results were comparable to those obtained with conventional extraction. Overall**,** the tendency of the changes in extractability as a function of the used parameters (time of EA, salt concentration, type of configuration) was the same for both tested current intensities. According to the alkalinity studies the titratable alkalinity of the electro-activated solutions during 30 min increased by 3 times compared to the electro-activated solution during 10 min. At the same time, it was observed that electro-activation during 60 min increased the solution alkalinity by 2 times compared to the solution which was electro-activated during 30 min. However, the difference in the amount of proteins extracted by each of these solutions was not significant. Considering the pH of the extraction medium as a key factor these results become logic. Apparently, the protein extractability has low sensitivity to pH changes within the region of pH 7–10 and the increase of the extractability is observed at pH values higher than 10. Indeed, even if napin fraction has isoelectric point close to pH 10–11, this did not affect the extractability because the other fractions were highly extracted under these pH conditions. Thus, the pH of the solution electro-activated was maintained around 7 during 10 min of extraction and the values obtained for the total dry matter extractability were 26.29 ± 0.25% in

0.01 M NaCl, 31.18 ± 1.20% in 0.1 M NaCl, and 32.75 ± 2.59% in 1 M NaCl. Conventional extraction held at pH 7 gave 24.84 ± 0.93%, 28.41 ± 1.88%, and 31.18 ± 1.89% for the same NaCl concentrations. However, the electro-activated solution obtained during 30 min at 300 mA maintained its pH around 8 and allowed to extract 27.72 ± 0.92%, 30.82 ± 0.61%, and 32.07 ± 2.42%, whereas the results for the conventional extraction held at the same pH 8 were 23.28 ± 0.90%, 25.30 ± 0.52%, and 27.78 ± 0.27% which were lower. Finally, the 60-min electro-activated solution with a pH 9–10 gave extraction yields of 33.82 ± 0.59%, 36.10 ± 1.24%, and 38.06 ± 0.13% which were substantially higher in comparison with conventional extraction carried out at pH 10 (23.67 ± 0.19%, 27.98 ± 0.47%, and 29.97 ± 1.69%). These results supported the hypothesis that electro-activation can enhance the reactivity of the solutions and their extraction ability by facilitating the break of the entrapping vegetable tissue and by the way increasing the dry matter release in the surrounding aqueous medium. An increase in protein extractability with an increase in salt concentration is due to the salting-in effect for conventional extraction and for the solutions obtained by electro-activation. Alkalinity of the solution increased with an increase in salt concentration (Gerzhova et al. 2015a) also increasing the protein extractability. The positioning of the anion and cation exchange membranes in the electro-activation reactor also significantly affected the protein extractability by the electro-activated solutions. Briefly, an anion exchange membrane which was placed in the cathodic chamber, separating it from the central section (Fig. 4.1), allowed the migration of hydroxyl ions, responsible for the alkalinity into the neighboring compartment. However, under some electrochemical conditions, in the cathodic chamber a zone of depletion (desalting) can be created and can lead to water dissociation on the membrane in order to supply the deficiency of electric current carriers. Thus, a generation of H^+ ions in the cathodic compartment limited the increase in alkalinity by neutralizing OH^- ions. This was especially pronounced when 0.01 M NaCl concentrations were used. With an increase in salt concentration the effect of water dissociation decreased which is supported by an increased alkalinity and the higher amount of extracted proteins. Also, the water dissociation decreased because the accumulated Na^+ ions can act as electric current carriers. When 1 M NaCl concentration was used, the effect of ion migration through the membrane was not significant for such high salt concentration. Regarding the canola protein isolates, the reported results indicated that there was no significant difference between the yields of the proteins extracted under conventional conditions at pH 10 and those produced by using electro-activated solution produced during 60 min of electro-activation. High proteins purity was reported for both methods, supporting the hypothesis that electro-activated solutions were successfully used to extract canola proteins and that they can be used to substitute NaOH which need special handling conditions since at the industrial scale it is purchased in a concentrated form and must be stored under strictly controlled conditions. Thus, the environmental impact of using electro-activated solution can be expected to be substantially better that the conventional process requiring high amounts of chemicals.

4.4.2 Soy Proteins Extraction

Gerliani et al. (2019c) studied a process of protein-carbohydrate extraction from soybean meal by using electro-activated aqueous solutions as extracting media (Gerliani et al. 2019c). Different aqueous NaCl solutions were electro-activated in the cathodic section of a three-compartmental electro-activation reactor. The compartments were separated by appropriate disposition of anion and cation exchange membranes to avoid a direct interaction between the anolyte (acidic electro-activated solution in the anodic compartment) and the catholyte (alkaline electro-activated solution in the cathodic compartment).

Electro-activation time and current intensity had significant effects on the alkalinity of the electro-activated solutions. The solutions electro-activated under 150 mA had the lowest titratable alkalinity compared to samples treated at 300 or 450 mA. Indeed, the first Faraday's law (Eq. 4.14) which states that the mass of the substance liberated on the electrode is directly proportional to the time and to the amount of electricity flowing through the electrolyte explains why time and current intensity have such effect (Damaskin et al. 2008).

$$m = Z \cdot I.t \tag{4.14}$$

where Z is a constant called electrochemical equivalent of the substance, I is the current intensity (Amperes), and t is the time (seconds).

Time and current-intensity correlations were observed in the cathodic compartment. The highest titratable alkalinity was generated at a current intensity of 450 mA, with a linear increase from 0.020 to 0.071 mol/L at 10 and 50 min, respectively. Likewise, short-time electro-activation for 10 min generated a low catholyte alkalinity for samples treated at 150, 300, and 450 mA (0.008, 0.010, and 0.020 mol/L), while longer time treatment (50 min) at the same current intensities (150, 300, and 450 mA) resulted in a higher titratable alkalinity (0.026, 0.052, and 0.071 mol/L). These results indicate that the solution alkalinity could be controlled by combining the corresponding electro-activation current intensity and treatment time. Thus, the electric-activated solutions can be used as a suitable replacement of chemical bases for different purposes, including extraction procedures. Thus, electro-activated solutions with given pH and alkalinity can be selected to correspond with the alkalinity of conventional solutions to carry out extraction from soybean meal (Gerliani et al. 2019a).

The total dry matter extractability is an important parameter to understand the amount of constituents that passes from the meal to the surrounding solution during the extraction process. The obtained results in the study of Gerliani et al. (2019b) showed that total dry matter extractability was dependent on the type of the electro-activated solution that was used (Gerliani et al. 2019c). In general, extraction carried out using the catholyte solution (alkaline) resulted in higher yields, being maximal at sample extracted by using the electro-activated solution at 450 mA for 50 min with a mean value of 46.77 ± 3.23%. The effect of current intensity on total dry matter extractability was more significant than the duration of electro-activation for the

catholyte solutions. For instance, samples treated with electro-activated catholyte solutions at 150 mA for 10 min yielded the lowest amounts of total dry matter (19.82 ± 1.27%). Moreover, this study showed that increasing the electro-activation duration had slight effect on the extractability, while increasing the current intensity up to 300 and 450 mA during the solution electro-activation resulted in a significant ($p < 0.001$) rise of extracted materials. This result indicated that increasing the current intensity resulted in more alkalinity in the electro-activated solution, which constitutes a key factor to increase the extraction yield from the used soybean meal. Indeed, the catholyte solutions electro-activated for 50 min resulted in low extraction yields of 24.51 ± 0.58%, at 150 mA but improved extracted amounts to 39.57 ± 2.12%, with increased current intensities to 300 mA and to 46.77 ± 3.23% with 450 mA.

The electro-activated catholyte solutions were highly potent for protein extraction, being maximal in sample electro-activated during 50 min under 450 mA (45.55 ± 2.77%). In spite, as was discussed previously the increasing of the electro-activation time and current intensity influenced the protein extraction ability of the catholyte due to a higher amount of the formed alkali (NaOH*eq*). Thus, samples extracted with catholyte that were obtained after 30 min of electro-activation contained more protein than samples extracted with catholyte after 10 min of electro-activation. The total protein content of the sample obtained with the catholyte 30 min-150 mA was 19.12 ± 1.38%, whereas one of the samples extracted with the 30 min-300 mA catholyte was 29.97 ± 0.90%. The amount of protein of the catholytic sample 30 min-450 mA was 38.24 ± 3.24%, indicating the high significance ($p < 0.001$) of the used electric current intensity on the amount of the formed NaOH following electro-activation of the NaCl solution. Moreover, samples obtained with catholyte that was electro-activated during 50 min tend to have a more important quantity of proteins. The amount of protein in the samples obtained with catholytes 50 min – 150 mA and 50 min–300 mA was 35.07 ± 0.93% and 37.97 ± 3.03%, respectively, whereas a total protein content of 45.55 ± 2.77% was obtained in the sample that was extracted by using a catholyte electro-activated at 50 min-450 mA. The conventional chemical alkaline extraction was conducted at pH 8, 9, 10, whereas the acid extraction was carried out at pH 2 and 3. In both extraction modes, different NaCl concentrations were added to the medium (0.01, 0.025, and 0.05 mole/L). The obtained results showed that both methods were effective in terms of extraction of soluble dry matter with significant qualitative and quantitative differences. The alkaline extraction was significantly more effective in terms of the total extracted dry matter and protein content in the extracted material with mean values of 39.9 ± 0.2 and 43.3% ± 2.9, respectively. The acid and alkaline extracts were characterized by high content (mg/100 g) of specific minerals such as potassium (334.93 ± 41.9), calcium (24.73 ± 10.3), and magnesium (35.3 ± 10.9). Regarding total sugars, the acid extracts contained more soluble sugars than the alkaline extracts. The obtained results were in good agreement with the known ability of alkaline solutions such as NaOH to extract high amounts of proteins, including some specific protein fractions of soybean meal that are mostly soluble in alkaline solutions due to the disruption effect on the soybean meal tissue (Pickardt

et al. 2009). Finally, the results obtained in the investigation by Gerliani et al. (2019b) showed that by using catholyte (alkaline solutions) the overall extraction of proteins from soybean meal can be significantly increased by controlling the electro-activation parameters (current intensity and treatment duration).

Samples obtained using catholyte as the alkaline extracting solutions showed good protein quality, with more intense bands on SDS-PAGE electrophoresis, and being maximal in catholyte sample electro-activated for 50 min. This result was in agreement with the higher amount of the formed NaOHeq following cathodic electro-activation of NaCl. Specifically, catholyte samples obtained with 10 min-150 mA, 10 min-300 mA, and 10 min-450 mA solutions have 5 bands with the average molecular weight of 64.6, 58.9, 44.9, 32.1, and 18.1 kDa. While, the sample 30 min-150 mA has 5 bands 63.5, 57.4, 43.8, 31.4, and 17.9 kDa, samples 30 min-300 mA and 30 min-450 mA had 8 bands ranging from 10.5 (MW corresponds to A_5 acid peptide of glycinin) to 63.4 kDa (MW corresponds to alfa subunit of beta-conglycinin) and 10.7 to 64.5 kDa, respectively. The latter sample has more intense bands in comparison with the samples 30 min-150 mA and 30 min-300 mA, which implies a better protein quality in the 30 min-450 mA sample. The extracted sample with the catholyte electro-activated at 50 min-150 min has 8 bands with molecular weights ranging from 10.9 to 66.9 kDa while 50 min-300 mA sample has 7 bands, and 50 min-450 mA has the 8 more intense bands (12.4, 19.8, 21.3, 29.4, 32.4, 45.4, 64.3, and 67.4 kDa). In addition, extraction during few hours seems to enhance the overall extraction (qualitatively and quantitatively) and thus can be used to obtain extracts with proteins having high molecular weights (data not shown) such as 100 kDa.

In previous work of Gerliani et al. (2019b) it was shown that the maximal molecular weight obtained in the basic conventional samples (pH 10 and alkalinity of 0.07 mol/L) was 93.5 kDa which corresponds to the molecular weight of lipoxygenase (Gerliani et al. 2019b, 2020a). For electro-activated samples obtained under the same alkalinity of 0.07 mol/L, the SDS-PAGE electrophoresis results showed that they did not contain lipoxygenase and the maximal molecular weight of the extracted fractions in these samples was 67 kDa which corresponds to molecular weight of alfa subunit of beta-conglycinin in the sample obtained by using the electro-activated alkaline solution at 450 mA during 50 min. In its turn, the presence of lipoxygenase in conventional samples of soybean meal extracts plays a role in oxidation of polyunsaturated fatty acids which forms a lipohydroperoxide; the primary product of lipoxygenase action (Murphy 2008). Moreover, often lipoxygenase causes beany and grassy off-flavors in foods that contain soy protein. In order to remove lipoxygenase, which gives these off-flavors, the product should be heated (Murphy 2008). However, none of the electro-activated samples treated in the same way as conventional samples contain this enzyme which allows avoiding the additional heat treatment procedure (Gerliani et al. 2019c, 2020b).

The conventional extract obtained at pH 8 had the highest water holding capacity (400 ± 7 g/100 g), while the lowest was that of samples extracted under pH 3. Extract obtained using electro-activated solution Anolyte_300mA-30 min had water holding capacity value of 25 ± 1 g/100 g. The oil absorption capacity was the highest

for samples extracted under alkaline conditions whatever the extraction mode used with values of 5.50 ± 0.54 to 6.85 ± 0.62 mL/g. The foaming capacity of the conventional extracts was higher compared to those extracted by electro-activation with maximal value of 52% for the conventional sample obtained at pH 9, whereas the maximal foaming capacity of 28% was observed for the electro-activated sample obtained by using Anolyte_450mA-50 min. Electro-activated samples showed higher emulsifying properties. Conventional extracts showed higher antioxidant activity (92.31 ± 1.5%) than those obtained by electro-activation (47.46 ± 0.94%). This difference in the antioxidant capacity could be attributed to the fact that electro-activation extraction was more efficient in eliminating the residual phenolic compounds in the meal. Indeed, most of the antioxidant capacity of the plant extracts are mostly attributed to these phenolic molecules.

4.5 Use of Extracted Proteins in Food Matrices

Electro-activated solutions were used to extract a protein-carbohydrate mixture from defatted soybean meal and the obtained extract was successfully used to produce a beverage (Gerliani et al. 2019c). The study reported by Gerliani et al. (2019b) aimed to investigate the physico-chemical, functional properties, and behavior during storage of plant extract-based beverages which were made by using complex extracts obtained from soybean meal by using electro-activated solutions (catholytes) as extracting agents. The aqueous solutions were first electro-activated in the cathodic compartment of the electro-activation reactor and then used as extracting agents. Different electro-activated solutions were prepared under varied electric current intensities and treatments duration. These solutions were characterized by different pH values and total titratable alkalinity. The detailed results can be found in the work of Gerliani et al. (2020a, b). The authors also reported that all beverages contain 0.6% whey powder. The soybean meal extract-based beverages were characterized by protein profiles with different particle size distribution. The viscosity of the most interesting beverage samples in terms of visual appearance and stability during refrigerated storage (absence of sedimentation) was low and the beverages showed a shear-thinning behavior. The turbidity and Zeta-potential measurements showed that the obtained beverages were sufficiently stable in the interval of pH 3–10. Even if some sedimentation was observed, the authors reported that a simple shaking allowed the system to recover homogeneous consistency. Moreover, solubility of the soybean meal extracts in the prepared beverages was high, with an average solubility value of 97.50%. Also, some beverage samples showed high foaming capacity with mean values of 98 ± 70% and 80 ± 50%, respectively. Also, depending on the used conditions, some of the beverage samples showed high water absorption capacity with an average value of 530 g/100 g. Moreover, the authors affirmed that after thermal coagulation assay to test the protein stability against heat treatment and their eventual stability, the obtained results showed that protein recovery values in the precipitate varied from 56.50% to 98.50%. This variation can be attributed to the molecular weight and thermal stability of the different protein fractions of the used

extract. This result supports the feasibility of pasteurizing the beverages with a minimal risk of protein precipitation following the heat treatment because the protein is well stabilized in the aqueous phase of the beverage. Also, the stability of the beverages made with the use of soybean extract can be assured by adding some amount of soluble polysaccharides to enhance their stability in suspension without risk of aggregation and precipitation. This stability can be achieved by exploiting the potential of utilizing interfacial complexes, formed through the electrostatic interactions of proteins and polysaccharides since proteins carry different positive and negative charges that can bind to some polysaccharides which also carry different electric charges on their surface, especially negatively charged functional groups (Harnsilawat et al. 2006; Zamani et al. 2020; Wei et al. 2020).

In another work, canola proteins extracted by electro-activated solutions were incorporated to gluten-free biscuits made from blend of rice and buckwheat flours (Gerzhova et al. 2016). The substitution level varied between 3 and 9% (w/w) of the rice flour in the blend. Physical and textural properties were significantly influenced by these proteins. Isolates and concentrates of proteins do not behave in a similar way. A significant increase in biscuit diameter was noted for all the supplemented samples. An increase in thickness was observed for the samples added with protein concentrate, whereas the spread ratio decreased for all the samples compared to the control without canola proteins. The hardness of biscuits decreased with the addition of the proteins as compared to the control. The changes in biscuit hardness or fracture strength were in line with the changes in dough hardness. Biscuits containing protein concentrate had lighter, more aerated texture as shown by scanning electron microscopy with lesser amounts of gelatinized starch, indicating improved gas-holding capacity and more stabilized structure. Protein-supplemented biscuits had lower moisture and water activity (Fig. 4.2).

A protein-carbohydrate complex was extracted by using alkaline electro-activated solution from canola meal (Salah et al. 2019). The study of Salah et al. (2019) aimed at developing a gluten-free food matrix by incorporating canola proteins in white rice flour bread formulation. This matrix offers to persons intolerant to gluten a product with good quality such as including enhanced mass volume, honeycomb structure, attractive color, and good nutritional value. In the first step, five formulations were tested, namely control-1 (100% wheat flour), control-2 (100% rice flour), rice flour +3% canola extract, rice flour +6% canola extract, and rice flour +9% canola extract. At each single manufacturing step, the initial and final products have been characterized using different techniques including volume expansion, temperature profile during fermentation and cooking, pH (acidity), water loss, mass volume, colorimetric analysis, total protein content, and texture profile analysis. At the second step, two independent variables were studied: addition of shortening (1, 2, 3%) and xanthan gum (0.5, 1, 1.5%) to improve the mass volume of the loaf obtained by the best formulation among the five tested formulations in the first step. Thereafter, correction attempts have been made to the obtained products by adding sodium bicarbonate (0.5, 1 and 1.5%) and canola oil (1, 2, 3%) instead of shortening. Bread making tests showed different mass volumes and honeycomb structures of lower quality compared to those of control-1 bread (2.52 mL/g), but significantly higher

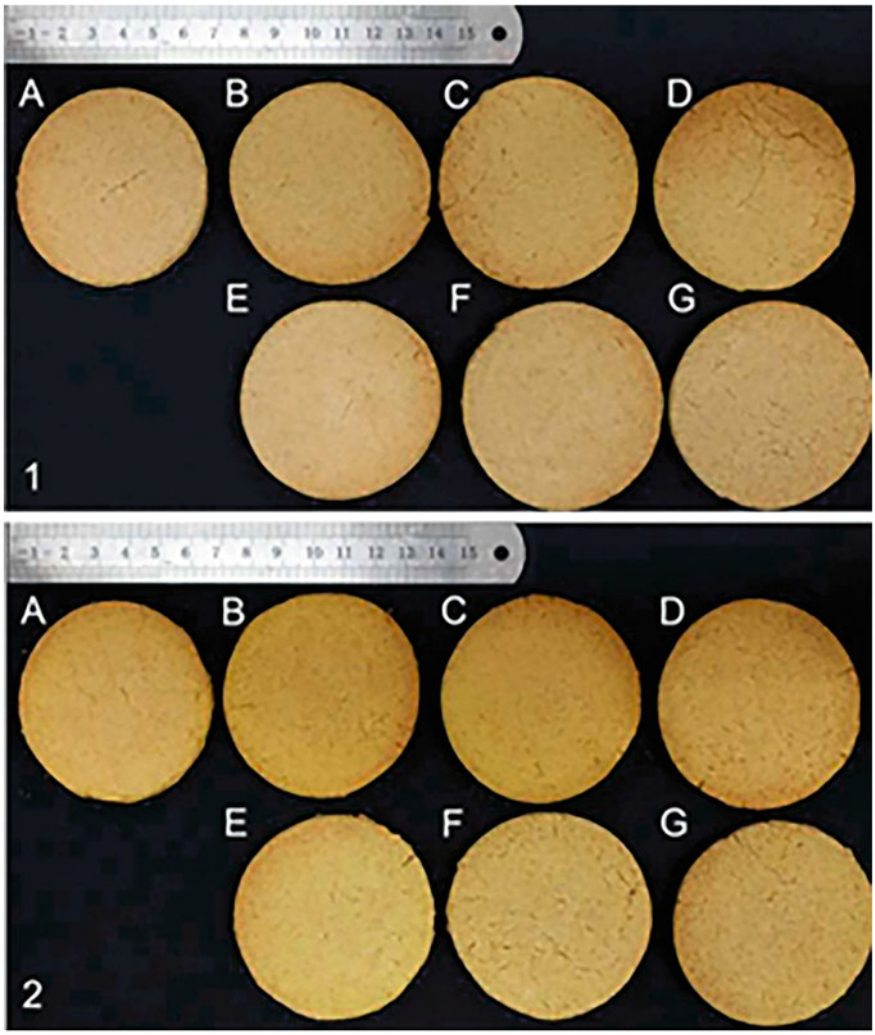

Fig. 4.2 Cookies prepared with a blend composed of white rice and dark buckwheat flours. (1): Rice and green buckwheat flours. (2) With the incorporation of canola protein concentrate (EA-CPC) and isolate (EA-CPI): (A) Control; (B) 3% EA-CPC; (C) 6% EA-CPC; (D) 9% EA-CPC; (E) 3% EA-CPI; (F) 6% EA-CPI; (G) 9% EA-CPI. Adapted from (Gerzhova et al. 2016)

than those of control-2 (1.42 mL/g). The highest mass volume of 1.78 (mL/g) was obtained with the bread formulation containing 6% canola meal extract. In summary, the study of Salah et al. (2019) demonstrated the positive impact of canola meal extract, rich in proteins, incorporation into white rice flour-based gluten-free bread. These results can contribute to the progress of research focusing on substituting gluten by other proteins having good techno-functional properties.

4.6 Concluding Remarks and Perspective

Electro-activation, a branch of applied electrochemistry, studies the modifications of the activity and reactivity of aqueous solutions following adequate treatment by an external electric field under specific reactor configuration and treating conditions. Even if all the known phenomena driven by an electric field in an aqueous media occur simultaneously in such a reactor, electro-activation deals only and specifically with its impact on the activation energy involved in specific chemical reactions. Thus, when electro-activation process is carried out, it is necessary to consider other electrochemical phenomena such as electrolysis, electrodialysis, electrocatalysis, and anion-cation transport through ion selective membranes. Nevertheless, these aforementioned phenomena are only concomitant to electro-activation but do not represent the electro-activation itself.

Electro-activation, as science and as a unit operation in different engineering processes, has a merit to be studied in depth because it can be used to improve and/or enhance different technological processes with real potential positive impact on environment protection by contributing to reduce many input materials such as extracting acids and alkaline solutions. It can be easily used to produce in place the required acids and bases that can be used in different technological processes, a fact that offers a possibility of eliminating the necessity of acquiring concentrated acids and alkaline solutions.

The use of electro-activation to produce protein-rich extracts which can be used as ingredients in different foods offers many advantages. From composition and techno-functional properties, extracts obtained by using electro-activation are of good quality and can be used in liquid and solid foods. Moreover, they have generally very good solubility which is an important factor for their eventual bioavailability. Furthermore, the use of electro-activation for alkaline extraction and acidic precipitation can be combined for a dual alkaline-acid extraction to improve the composition and functionalities of the extracts. From environmental consideration, electro-activation technology can be easily integrated in a process design of extraction unit operations to avoid pollutant effluents by substituting extracting and precipitating chemicals by their equivalent electro-activated solution that can be produced in-place depending on the process requirements and needs.

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Emerging Solvent Extraction Technologies for Plant Protein Extraction: Aqueous Two-Phase Extraction; Deep Eutectic Solvent; Subcritical Water Extraction

5

Martin Mondor and Alan Javier Hernández-Álvarez

Abstract

The plant protein market has been booming for several years now; consumers are increasingly looking for alternative sources to animal protein. Nowadays, plant protein concentrates/isolates are mainly produced by the alkaline extraction-isoelectric precipitation process. This process has a high productivity but is not so environmentally friendly due to the generation of a large volume of effluent following the isoelectric precipitation of the proteins. Under some conditions, the functional properties of the extracted proteins can also be negatively impacted. However, some innovative solvent extraction technologies for plant protein extraction (Aqueous two-phase extraction; Deep eutectic solvent; Subcritical water extraction) are emerging and they have high potential to preserve the functional properties of the extracted proteins. Aqueous two-phase extraction, deep eutectic solvent, and subcritical water extraction are also considered green extraction technologies. In this chapter, the basic principles of each technology, as well as their impact on protein extraction and functionality will be presented.

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Keywords

Aqueous two-phase extraction · Deep eutectic solvent · Subcritical water extraction

5.1 Introduction

The global plant-based protein market is expected to account for US$17.4 billion by 2027, growing at a compound annual growth rate of 7.3% between 2022 and 2027 (MarketsandMarkets n.d.). The growth of this market can be attributed to the growing trend of veganism and manufacturer involvement in plant-based proteins driven by strong retail sales in plant-based products. The main plant protein sources for food and feed are soybean, wheat, and corn (Sari et al. 2015). However, new alternative sources are finding their way into the market, including barley, bean, canola, chickpea, flax, hemp, lentil, pea, quinoa, and rice (Özbek and Bilek 2018). Plant proteins can be extracted directly from the plant or from plant by-products. Several sources of plant proteins are from different industrial waste materials (Aiking 2011). The redirection of plant by-products, which are usually used as animal feed livestock, to human consumption helps to preserve the environment, ensure food security, and support the sustainability of food systems (Pojić et al. 2018).

Plant proteins can be extracted/purified by various processes, and the selection of the process will depend on the type of ingredient (flour; concentrate; isolate; hydrolysates) that is suited (Mondor and Hernández-Álvarez 2022). Alkaline extraction-isoelectric precipitation of the proteins is the process the most widely applied at the industrial scale. It consists in the extraction of the proteins at alkaline pH (usually between 8 and 11) under agitation, followed by the removal of the insoluble matter by centrifugation, and by the isoelectric precipitation of the proteins contained in the supernatant by the addition of acids to decrease the pH to the isoelectric point of the proteins, which is around 4.5 for most plant proteins. The proteins are then recuperated by centrifugation and they are resolubilized in water at pH 7 before being dried to obtain a protein concentrate or isolate (Mondor and Hernández-Álvarez 2022). Despite the fact that the alkaline-isoelectric precipitation process has a high productivity and that it can be easily scaled, it also generates effluents that may have a negative impact on the environment, and it is known to negatively impact the functionality of the proteins, especially the protein's solubility after rehydration (Mondor et al. 2004). For that reason, various alternative processes have been considered for the extraction/purification of plant proteins (Mondor and Hernández-Álvarez 2022). Among these processes there are emerging green solvent extraction technologies, in particular aqueous two-phase extraction (ATPE), deep eutectic solvent (DES) extraction, and subcritical water extraction. This chapter provides a review of the aforementioned solvent processing technologies used to extract plant proteins and discusses their impact on the main plant protein sources.

5.2 Aqueous Two-Phase Extraction

Conventional ATPE is based on the formation of an aqueous two-phase system (ATPS) when two water-soluble polymers or a salt and a polymer are dissolved in water beyond a critical concentration at which two immiscible phases form (Raghavarao et al. 1995). The dependency of phase composition on the polymer concentrations at given temperatures, pressures, and molecular weights of the polymers is often graphically displayed in a phase diagram where the one-phase region is separated from the two-phase region by the binodial curve, as shown in Fig. 5.1 (Raghavarao et al. 1995). Dextran and polyethylene glycol (PEG) were among the first polymers considered for ATPS protein extraction (Diamond and Hsu 1990). Over the years, a wide variety of ATPS phase-forming components such as thermo-sensitive polymers, light- or pH-sensitive polymers, short aliphatic alcohols, organic solvents, and ionic liquids have been studied to develop more sustainable and effective separation and purification technologies based on the ATPS technique (Lee et al. 2017). For ATPS based on ionic liquids, conventional ionic liquid cations were the imidazolium-based cations, and the anions were halides or tetrafluoroborate (Lee et al. 2017). Today, ionic liquid cations that are used for protein extraction include: cholinium cation, ammonium cation containing an oligoethyleneglycol or oligopropyleneglycol unit, hydroxyl-functionalized ammonium cation, quaternary

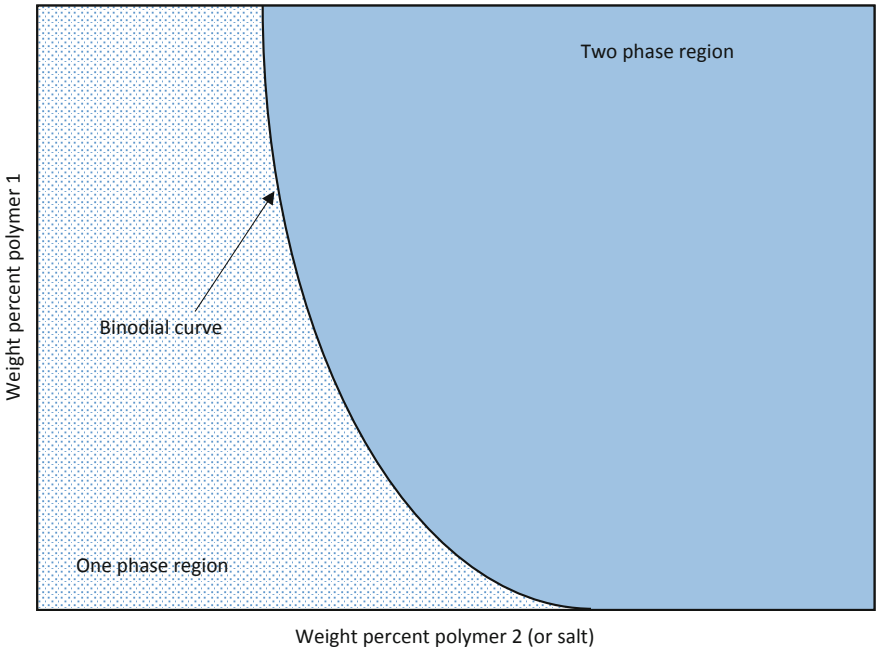

Fig. 5.1 ATPE phase diagram

ammonium or phosphonium, and guanidinium cation. Carboxylic acids, amino acids, and biological buffers are used as ionic liquid anions (Lee et al. 2017).

For protein extraction, a suitable amount of each ATPS phase-forming solution is mixed with the aqueous solution containing the target protein. In an ideal system, when phase separation is achieved, the target protein is concentrated in one of the two immiscible phases. However, in practice, the target protein will be distributed between the two immiscible phases. The ratio of equilibrium concentration of a protein in the top and bottom phases is defined as the partition coefficient (K) and it can be estimated as follows (Santhi et al. 2020):

$$K = \text{Protein concentration in top phase}/\text{Protein concentration in bottom phase}$$

Another parameter of importance to characterize an ATPE process is the selectivity, which characterizes the extent to which the system can separate the target protein from an unwanted protein also present in the protein solution. The selectivity (α) can be estimated from the partition coefficient of the target and unwanted proteins as follows (Gu and Glatz 2007):

$$\alpha = K_{\text{target protein}}/K_{\text{unwanted protein}}$$

The recovery of the target protein in the top phase can be estimated as follows (Santhi et al. 2020):

$$R_{\mathrm{T}}\ (\%) = C_{\mathrm{T}}{}^{*}\ V_{\mathrm{T}}/(C_0{}^{*}V_0)^{*}\ 100$$

where R_{T} is the recovery of the target protein in the top phase, C_0 is the concentration of the target protein in the total phase, V_0 is the volume of the total phase system, and C_{T} and V_{T} are the concentration of the target protein in the top phase and the volume of the top phase, respectively.

A similar equation can be applied for the recovery of the target protein in the bottom phase (Santhi et al. 2020):

$$R_{\mathrm{B}}\ (\%) = C_{\mathrm{B}}{}^{*}\ V_{\mathrm{B}}/(C_0{}^{*}V_0)^{*}\ 100$$

where R_{B} is the recovery of the target protein in the bottom phase, and C_{B} and V_{B} are the concentration of the target protein in the bottom phase and the volume of the bottom phase, respectively.

Another parameter of interest is the tie line length (TLL), which characterizes the compositional differences between the two phases (Gu and Glatz 2007):

$$\mathrm{TLL} = \left(\left(\Delta C_{\text{Phase – forming solution 1}}\right)^2 + \left(\Delta C_{\text{Phase – forming solution 2}}\right)^2\right)^{0.5}$$

where $\Delta C_{Phase-forming\ solution\ 1} = C^{Top}_{Phase-forming\ solution\ 1} - C^{Bottom}_{Phase-forming\ solution\ 1}$

$$\Delta C_{Phase-forming\ solution\ 2} = C^{Top}_{Phase-forming\ solution\ 2} - C^{Bottom}_{Phase-forming\ solution\ 2}$$

where C_i^j is the concentration of component i (Phase-forming solution 1 or phase-forming solution 2) in phase j (Top or bottom phase).

Different factors can influence the efficiency of the ATPE process, including the choice of the phase-forming components and their concentration in the aqueous phases, pH, temperature, phase ratio, and the way the solution containing the target protein is mixed or put into contact with the phase-forming solutions (Raghavarao et al. 1995; Gu and Glatz 2007). Raghavarao et al. (1995) have indicated that for polymer phase-forming solutions, the molecular weight of the polymers has an effect on the partitioning of the target protein, because it affects the phase compositions. An increase in molecular weight of the polymers of one phase will lead to the target protein being partitioned strongly in the other phase (Albertsson et al. 1987). However, this effect depends on the molecular weight of the target protein, with negligible effects for protein with molecular weight less than 10,000 Da (Albertsson et al. 1987). Regarding the effect of the pH, the partitioning of protein in ATPE is affected by its net charge, which in turn depends on the pH of the solution. Change in pH may also lead to conformational changes, which lead to changed partitioned behavior of the proteins (Raghavarao et al. 1995). The influence of temperature is an indirect one. The shape of the binodial curve and protein partitioning tends to change with temperature. Also, as aforementioned for the pH, temperature change may lead to conformational changes due to protein denaturation, which in turn causes changes in partition behavior (Raghavarao et al. 1995).

ATPE is considered as an environmentally-friendly process since, in general, both phases contain more than 80% water (Santhi et al. 2020) and the system usually consists of components that are generally recognized as safe (GRAS). Another interesting feature of aqueous two-phase systems is that partitioning of proteins, in general, does not depend on their concentration and volume of the system, making the process easily scalable. An overview of ATPE processes for the isolation of plant proteins from various sources is presented in Table 5.1.

5.3 Deep Eutectic Solvent Extraction

Deep eutectic solvents consist of chemical components which, separately, are solids with high melting points, but are liquids at relatively low temperatures when mixed together at appropriate molar ratios. The chemical components associate with each other through hydrogen bond interactions (Wahlström et al. 2017). Deep eutectic solvents are emerging as a promising and environmentally-friendly alternative to conventional organic solvents for the extraction of various biological compounds (Lin et al. 2021). Deep eutectic solvents are regarded as a new class of green solvents because of their unique properties, such as easy synthesis, low cost, low volatility, high dissolution power, high biodegradability, and feasibility of structural design (Yucui et al. 2018). Based on Ivanovic et al. (2020), DESs can be divided into four

Table 5.1 Overview of aqueous two-phase extraction process for the isolation of plant proteins from various sources

Protein source	Protein content of starting material	Protein recovery (based on the protein content of the starting material, unless noted)	Main finding	Reference
Aloe leaves	n.a.	Up to 95.85% in the ionic liquid-rich phase (based on the amount of protein in the crude aloe polysaccharide solution)	1.0 g [Bmim]BF4, 3.0 mL water, a given amount of salt, and 1.0 ml Aloe polysaccharide solution were mixed together. Polysaccharide can be extracted in the salt-rich phase with high extraction efficiency, while the majority of proteins were extracted in the ionic liquid-rich phase.	Tan et al. (2012)
Coconut whey	1.10 ± 0.02 mg/ml	Up to 92.65% (w/w)	PEG 6000/potassium phosphate system (5%/12% w/w, 0.2 volume ratio, and pH 8.0) was observed to be the most suitable system, resulting in a recovery of 92.65% (w/w) in the salt-rich phase.	Santhi et al. (2020)
Corn (clarified extract; unclarified extract; corn solids)	n.a.	Up to 100% for cytochrome c	The aim of this study was to recover recombinant proteins using lysozyme, ribonuclease A, and cytochrome c as model proteins. The combination of PEG MW of 1450 with 8.5 wt.% NaCl addition (Na_2SO_4 as the phase-forming salt) provided for complete recovery of cytochrome c in the lower phase.	Gu and Glatz (2007)
Rubisco (purity ~80%)	n.a.	Up to 98% (calculated as the	Ionic liquid-based aqueous two-phase	Desai et al. (2014)

(continued)

Table 5.1 (continued)

Protein source	Protein content of starting material	Protein recovery (based on the protein content of the starting material, unless noted)	Main finding	Reference
		percentage of the amount of Rubisco in the top phase to the initial amount)	extraction of Rubisco using Iolilyte 221 PG and sodium potassium phosphate buffer was investigated.	
Soy extracts (supernatant obtained by the extraction of defatted soy flour at pH 8.0)	n.a.	n.a.	The storage proteins were present in the bottom phase of system Al (15% PEG 1000, ammonium sulfate, pH 7.5), but not detectable in the top phase.	Cole (1993)
Soy extracts (7S or 11S)	7S: 32 mg/cm^3 11S: 9.0 mg/cm^3	Up to 83% recovery of GUS	The aim of this study was to recover recombinant proteins using β-glucuronidase (GUS) as a model protein. A PEG 600/phosphate system comprising 14.5% (w/w) PEG, 17.5% (w/w) phosphate, a volume ratio equal to 1.0, and a pH of 7.0 resulted in the potential 83% recovery of GUS and an increase in purity of 4.5 fold.	Aguilar and Rito-Palomares (2008)
Tobacco leaves	n.a.	17.5 mg/g	Ground tobacco leaves were treated with 50 mM ABC (pH 8.0): 4% (w/v) 1-dodecyl-3-methylimidazolium chloride.	Li et al. (2020)

GUS β-glucuronidase, *n.a.* not available, *MW* Molecular weight, *PEG* polyethylene glycol

groups: (1) quaternary ammonium salt and hydrogen bond donor; (2) quaternary ammonium salt and metal chloride; (3) quaternary ammonium salt and metal chloride hydrate; (4) metal chloride and hydrogen bond donor. Among the four groups,

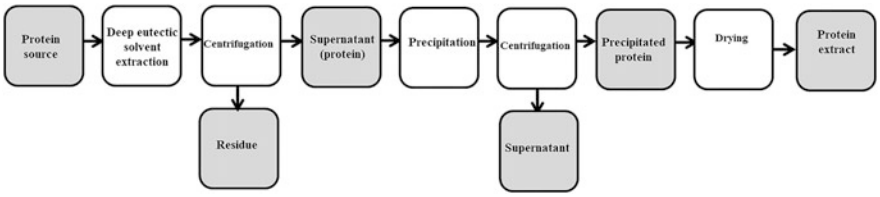

Fig. 5.2 DES extraction process

the DESs consisting of quaternary ammonium salt and hydrogen bond donor are the most commonly used. The quaternary ammonium salt is usually choline chloride, while the most common hydrogen bond donors are polyalcohols and urea. Different factors will affect the application and the performance of the DESs for the extraction of biological compounds, including its viscosity, conductivity, density, and polarity (Dai et al. 2013; Mišan et al. 2020).

For extraction purposes, the selected DESs are mixed with the material containing the compounds of interest for the extraction. The compounds of interest are then extracted for a given period of time under heating and agitation. Following the extraction step, the mixture is cooled to room temperature and is centrifuged to recover the supernatant containing the compounds of interest. The aforementioned extraction step can be repeated on the precipitated material recovered during the centrifugation step using fresh DESs. Once all the supernatants have been pooled together, the compounds of interest are precipitated and recovered by centrifugation (Grudniewska et al. 2018; Liu et al. 2017; Yue et al. 2021). The DES extraction process is illustrated in Fig. 5.2.

DES extraction has been used for various applications, including the fractionation of lignocellulose components (Bai et al. 2020; Hong et al. 2020; Zhai et al. 2020), the extraction of bioactive components from plants (Bajkacz and Adamek 2018; Ivanovic et al. 2020; Shang et al. 2019; Vázquez-González et al. 2020), and the extraction of proteins (Grudniewska et al. 2018; Lin et al. 2021; Liu et al. 2017; Mišan et al. 2020; Parodi et al. 2021; Wahlström et al. 2017; Yue et al. 2021).

5.3.1 Impact of DESs on Protein Extraction and Functionality

Some studies have compared the performance of DES extraction with that of conventional alkaline extraction (Lin et al. 2021; Wahlström et al. 2017). In their work, Lin et al. (2021) have extracted the proteins from the tips of bamboo shoots (TBS), as well as from its processing by-products (basal bamboo shoot (BBS) and sheath) using choline chloride (ChCl) and levulinic acid as DES. They varied the following extraction parameters: ChCl/levulinic acid molar ratio (2–6), solid-to-liquid ratio (30–70 mg/ml), temperature (40–80 °C), time (10–50 min), and water content (0% to 40% v/v). Conventional alkaline extraction was also applied for comparison by applying a solid-to-liquid ratio of 30 mg/ml at 40 °C for 10 min after the addition of 0.2% (w/w) NaOH. The supernatant was collected after

centrifugation and was used for protein quantification. The optimum DES extraction conditions were ChCl/levulinic acid molar ratio of 6, solid-to-liquid ratios of 30 mg/ml for TBS and 70 mg/ml for BBS and sheath, temperature of 80 °C, time of 50 min, and water content of 40%. The corresponding protein contents of the extracts were 39.16 ± 1.22 mg/g dry basis (d.b.) for TBS, 15.46 ± 0.30 mg/g d.b. for BBS, and 9.54 ± 0.17 mg/g d.b. for sheath, which was significantly higher than for the conventional extraction for TBS (23.88 ± 1.10 mg/g d.b.) and similar for BBS (16.99 ± 0.14 mg/g d.b.) and for sheath (8.29 ± 0.88 mg/g d.b.). However, one may note that, for a better comparison, it would have been of interest to perform the conventional extraction under similar conditions as for the DES extraction (solid-to-liquid ratio of 30 mg/ml at 80 °C for 50 min).

In another work, Wahlström et al. (2017) have compared the performance of DES extraction with that of conventional alkaline extraction for the recovery of proteins from brewer's spent grain. DES that were considered in their study were NaAcO, KAcO, and Na formate in different ratios with urea (NaAcO: Urea (1:2); NaAcO: Urea (1:3); KAcO: Urea (1:2); KAcO: Urea (1:3); Na formate: Urea (1:2); NaFormate: Urea (1:3)). All DES extractions were done with 10 wt% water added to the DES and at 80 °C for 20 h. DES extraction with choline chloride: urea (1:2) was also carried out for comparison. Conventional alkaline extraction was carried out at pH 11, 80 °C for 1 h. In a first series of experiments, a filtration step was performed to separate the residue from the extract, followed by washing the residue on the filter with water. Results indicated that the protein extraction yield was low for the conventional alkaline extraction process at 19%. The chlorine chloride: urea (1:2) system resulted in a protein yield of 23% and the 90 wt% NaAcO: urea (1:2) in a protein yield of 51%. Following the protein extraction, the extracts were dialyzed and freeze-dried to obtain protein concentrates. The chlorine chloride: urea (1:2) system resulted in a protein content of 52.0%, and the 90 wt% NaAcO: urea (1:2) resulted in a protein content of 54.7%. The protein extraction yields were improved following a modification to the washing step, which consisted in washing the residue in hot water, under stirring, and then filtering the residue and combining the washing liquor with the initial extract. After 4 h of extraction at 80 °C, high protein extraction yields were obtained: 79% with 90 wt% NaAcO: urea (1:2), 78% with KAcO: urea (1:3), and 74% with 90 wt% NaAcO: urea (1:3).

In their work, Liu et al. (2017) have combined DES extraction with an ultrasonic–microwave system to extract protein from pumpkin seeds (*Cucurbita moschata*). Deep eutectic solvents consisted of choline chloride and PEG 200 at different molar ratios (9:1; 3:1; 3:2). Defatted seed powder was extracted with the choline chloride: PEG 200 DES and was submitted to microwave and to ultrasound (240 W). Extracted proteins were recovered in the supernatant after centrifugation. The effect of the following experimental parameters on the protein extraction was studied: Liquid to solid ratio 10–40 ml/g, 50–190 W microwave power, and 30–60 °C temperature. Results showed that the optimal extraction conditions were PEG 200-based DES concentration of 28% w/w, liquid to solid ratio of 28 ml/g, microwave power of 140 W, and extraction temperature of 43 °C. This resulted in an extraction yield of 93.95 ± 0.23%.

Grudniewska et al. (2018) have extracted proteins from evening primrose cake and from rapeseed cake using a glycerol−choline chloride DES. Each ground cake (5 g) was mixed with 45 g of DES, and the mixture was stirred at 500 rpm at 60, 100, or 140 °C for 2 h. The supernatant containing the proteins of interest was recuperated following a centrifugation step. The proteins were precipitated by the addition of 250 mL of deionized water and incubation of the mixture at 4 °C for 12 h and they were recuperated by centrifugation. The protein content of the evening primrose extracts was between 40% and 50%, which was higher than for the starting cake (~28%). Similar results were obtained for the rapeseed extracts, with 36% to 48% proteins, compared to ~32% for the rapeseed cake. For the evening primrose, the protein yield varied between 8.4% and 34.2%, while it varied between 11.5% and 19.9% for the rapeseed cake. Optimum protein yield was obtained at 140 °C.

Yue et al. (2021) have studied 18 DESs consisting of choline chloride with butanediol isomer (1,2-butanediol, 1,4-butanediol, or 2,3-butanediol) in the absence or presence of water at different molar ratios. Combinations of choline chloride with 1,2-butanediol, 1,4 butanediol, or 2,3 butanediol, at a molar ratio of 1:3, with and without water, were found to have good oat protein extraction performance. Protein content of the extract varied between 37.51% ± 0.05% and 57.41% ± 0.70% while the protein recovery varied between 10.90% ± 0.21% and 42.92% ± 0.52%. Extraction with choline chloride-2,3-butanediol with water (molar ratio: 1:3:1) for 120 min was optimal, both in terms of protein content (57.41% ± 0.70%) of the extract and protein recovery (42.92% ± 0.52%). Precipitated proteins extracted with these conditions for 120 min also showed the better solubility and foaming capacity.

Parodi et al. (2021) extracted camelina, flax, and sunflower proteins from the corresponding residual press cakes obtained after oil extraction using DES (choline chloride with glycerol). The residual press cakes were ground to fine powder prior to the extraction and 5 g were mixed with 45 g of DES. The mixture was stirred at 60 °C or 90 °C for 2 h, followed by cooling of the mixture and recovery of the supernatant by centrifugation at 3000 rpm for 10 min. The residue was washed three times with 5 g of fresh DES and was centrifuged (4000 rpm, 10 min) to recover the supernatant which was pooled with the original supernatant. Protein precipitation was achieved by addition of 250 ml of deionized water to the combined liquid DES and incubation at 4 °C for 16 h, followed by centrifugation at 4000 rpm for 10 min, washing of the precipitate with deionized water, and drying. Each extraction was performed in triplicate. Yield (mg/g cake) of the precipitate obtained after DES extraction was low for the sunflower proteins with values of ~7.5 at 90 °C and ~ 1.0 at 60 °C. The authors attributed these low yields to the high level of phenolic compounds present in sunflower cake. These polyphenols can interact covalently or non-covalently with the proteins and affect their extraction properties (Prigent et al. 2003). Yields observed for the camelina and flax proteins were significantly higher with values of ~35.5 at 90 °C and ~ 25.5 at 60 °C for the camelina proteins and ~ 34.0 at 90 °C and ~ 32.5 at 60 °C for the flax proteins.

5.4 Subcritical Water Extraction

Subcritical water is defined as hot water (100–374 °C) in a liquid state under high pressure (up to 22 MPa) (Zhang et al. 2019). Under these conditions, the dielectric constant of water decreases, and the hydrogen bonding structure is weakened. As a result, subcritical water is more efficient than water to solubilize apolar components (Álvarez-Viñas et al. 2021). Subcritical water extraction is cheap, easily scalable, and environmentally friendly. Subcritical water has been used to extract biological compounds such as proteins (Du et al. 2020; Ho et al. 2007; Kataoka et al. 2008; Khuwijitjaru et al. 2011; Lu et al. 2016; Ndlela et al. 2012; Pinkowska et al. 2014; Ramachandraiah et al. 2017; Sereewatthanawut et al. 2008; Sunphorka et al. 2012; Viriya-Empikul et al. 2012; Watchararuji et al. 2008; Wiboonsirikul et al. 2007, 2013; Zhang et al. 2019) and carbohydrates (Abaide et al. 2019; Vedovatto et al. 2021; Viriya-Empikul et al. 2012; Watchararuji et al. 2008; Wiboonsirikul et al. 2013). However, due to its high temperature, under some conditions, subcritical water will hydrolyze the proteins and carbohydrates. For peptide and amino acid production, subcritical water hydrolysis can be considered as a good alternative to enzymatic hydrolysis, since it is less expensive and faster (Álvarez-Viñas et al. 2021). An overview of subcritical water extraction process for the isolation of plant proteins from various sources is presented in Table 5.2.

The efficiency of the subcritical water extraction process will depend on different operational parameters such as mode of operation, solvent-to-solid ratio (v/w), temperature, and time of extraction (Álvarez-Viñas et al. 2021). Regarding the mode of operation, most studies were carried out in batch mode, but some studies were also performed in semicontinuous or continuous mode (Hwang et al. 2015; Khuwijitjaru et al. 2011; Sunphorka et al. 2012; Wiboonsirikul et al. 2013). Operation in continuous mode provides higher productivity than operation in batch mode (Álvarez-Viñas et al. 2021). The solvent-to-solid ratio can also have a significant impact on the efficiency of the subcritical water extraction process. In general, the protein extraction will increase with an increase in the solvent-to-solid ratio (Ndlela et al. 2012). However, the solvent-to-solid ratio may vary significantly as a function of the raw material, with values varying between 3 and 210 being reported in the literature (Álvarez-Viñas et al. 2021). A lower ratio will result in poor mixing and makes external mass transfer difficult, which in turn will be responsible for the poor protein extraction efficiency (Watchararuji et al. 2008). Regarding the impact of the temperature on the protein extraction efficiency by subcritical water, it is well known that the dielectric constant and the energy required to disrupt the solute–matrix interactions will decrease with an increase in temperature. Elevated temperature also decreases surface tension and viscosity of water, which improves the ability of water to dissolve various compounds (Ho et al. 2007). Consequently, an increase in temperature usually results in a higher extraction yield. However, the maximum operating temperature for subcritical water extraction can be dictated by the stability of the targeted compounds to be extracted. Above a given temperature, which will vary with the compounds of interest, thermal degradation can be observed, which can limit the maximum operating temperature for the extraction (Ho et al. 2007). The

Table 5.2 Overview of subcritical water process for the isolation of plant proteins from various sources

Protein source	Protein content of starting material	Protein content of extract	Protein recovery (based on the protein content of the starting material, unless noted)	Main finding	Reference
Brewer's spent grain	17.7 ± 0.1%	Up to 13.8% at 185 °C	Up to 78% of solubilized protein at 185 °C	Temperature was varied from 125 to 185 °C at a constant flow rate of 4 mL/min. Optimal extraction temperature was 185 °C.	Alonso-Riaño et al. (2021)
Brewer's spent grain	21.9%	36.7 ± 0.1%	7.2 ± 0.9%	Subcritical water extraction conditions: 200 °C, pH 7, 20 min. Protein contents of the extract and protein yield for conventional extraction (40 °C, pH 11, 20 min) were higher at 60.2 ± 0.7% and 21.4 ± 0.9%, respectively.	Du et al. (2020)
Flaxseed meal	33.31 ± 0.05%	n.a.	67.4 ± 0.8%	Optimal conditions for protein extraction were 160 °C, pH 9, and a solvent-to-solid ratio of 210 mL/g meal.	Ho et al. (2007)
Rapeseed cake	34.98 ± 0.26%	n.a.	Yield of amino acids of up to 135.9 g/kg of rapeseed cake	A maximum yield of amino acids of 135.9 g/kg of rapeseed cake was obtained at 215 °C after 36 min.	Pinkowska et al. (2014)
Deoiled rice bran	16.8%	Up to ~0,325 g/l of extract	n.a.	Subcritical water extraction conditions: 180–280 °C for 5 min. Maximum protein concentration in the extract was observed at 250 °C.	Hata et al. (2008)
Deoiled rice bran	18.56 ± 0.31%	n.a.	21.9 ± 2.6%	Subcritical water extraction conditions: 100–220 °C and 0–30 min. The highest yield of protein was obtained at 200 °C and 30 min.	Sereewatthanawut et al. (2008)
Deoiled rice bran	16.9%	n.a.	Up to 17.86 ± 0.63%	Subcritical water extraction conditions: 116–284 °C, 0–79 min, and 2–12 MPa. The highest yield of protein was obtained at 250 °C, 60 min, and 4 MPa.	Sunphorka et al. (2012)

Rice bran	Raw: 14.06% Deoiled: 15.53%	Raw: 9.7% to 10.6% Deoiled: 12.9% to 13.0%	n.a.	Subcritical water extraction conditions: 50–250 °C, 10–30 min, material-to-water weight ratio (1:5 and 2:5). For both raw and deoiled rice bran, the optimal yield was obtained at 220 °C for 30 min.	Watchararuji et al. (2008)
Deoiled rice bran	16.8%	n.a.	~10% to 50%	Subcritical water extraction conditions: 200–220 °C for 5 min. The highest protein yield was obtained at 200 °C.	Wiboonsirikul et al. (2007)
Full-fat soy flakes	Soy flakes: 35.9% w.b. Extruded soy flakes: 35.3% w.b.	n.a.	Soy flakes: 31% to 72% Extruded soy flakes: 27% to 73%	Subcritical water extraction conditions: Solids-to-liquid ratio (1:3.3–1:11.7), temperature (66–234 °C), and extraction time (13–47 min). Optimal protein extraction conditions were 1:10, 100 °C and 40 min for flakes and 1:7.5, 234 °C and 30 min for extruded flakes, respectively.	Ndlela et al. (2012)
Defatted soy meal	49.42 ± 4.40%	52 ± 3%	n.a.	Maximum protein content in the liquid extract was obtained at 225 °C.	Khuwijitjaru et al. (2011)
Soy meal	n.a.	~80%	59.3%	Subcritical water extraction conditions: 120 °C and 20 min. The yield obtained by conventional alkaline extraction (pH 9.0) and acid precipitation (pH 4.5) at 25 °C was only 16.4%, while the protein content of the extract was ~80%.	Lu et al. (2016)
Soy meal	Raw: 33.87% Deoiled: 43.55%	Raw: 15.1% to 16.6% Deoiled: 14.3% to 20.5%	n.a.	Subcritical water extraction conditions: 200–220 °C, 10–30 min, material-to-water weight ratio (1:5 and 2:5). For raw soybean, the optimal yield was obtained at 210 °C for 30 min and at 200 °C for 20 min for the deoiled soybean.	Watchararuji et al. (2008)

(continued)

Table 5.2 (continued)

Protein source	Protein content of starting material	Protein content of extract	Protein recovery (based on the protein content of the starting material, unless noted)	Main finding	Reference
Soy	Daewonkong cultivar: 39.4% Saedanbaek cultivar: 48.7%	n.a.	36% to 77%	Subcritical water extraction conditions: 150 to 250 °C and pressure of 22 MPa. Temperature of 190 °C resulted in the highest yield (70% to 77%).	Ramachandraiah et al. (2017)
Sunn hemp	n.a.	n.a.	Up to 74%	The maximum protein extraction yield of 74% was obtained at 240 °C with 28 w% of Na_2CO_3.	Nyankson et al. (2013)

time of extraction is another operating parameter that will significantly impact the extraction yield. In general, the yield of extraction will increase with an increase in the time of extraction until a plateau is reached (Ho et al. 2007; Pinkowska et al. 2014; Sereewatthanawut et al. 2008; Sunphorka et al. 2012; Watchararuji et al. 2008). Temperature and time should be optimized simultaneously, since both parameters can have strong interactions (Wiboonsirikul et al. 2013). For protein, the yield of extraction can be increased by increasing the temperature and/or the time of extraction. However, the proteins denature at high temperatures and they are decomposed to peptides and amino acids.

5.4.1 Impact of Subcritical Water on Protein Functionality

As aforementioned, under some conditions, subcritical water will hydrolyze the proteins and carbohydrates when used as extraction technology, and it is an interesting alternative to enzymatic hydrolysis for peptide and amino acid production. However, subcritical water can also be of interest to modulate the functional properties of plant proteins (Khuwijitjaru et al. 2011; Lu et al. 2016; Wang et al. 2019; Zhang et al. 2015, 2018).

In their work, Khuwijitjaru et al. (2011) have processed soy meal with subcritical water at 100, 125, 150, 175, 200, 225, and 250 °C for 5 min in a batch-type reactor. Treatment at 225 °C resulted in the extract with the highest protein content (52%) while the extract resulting from the treatment at 100 °C had less than 10% protein. All the extracts formed oil-in-water type emulsions with a different emulsifying activity index (EAI) and emulsion stability index (ESI). The highest EAI was obtained at 100 °C (~90 m^2/g), while the highest ESI was obtained at 250 °C (~30 min). Foaming properties were also assessed, and it was found that the extract at 175 °C was the one showing the highest volume of foam over 180 min.

Lu et al. (2016) have combined enzyme-assisted extraction with subcritical water treatment for the extraction of soy meal proteins. The soy proteins were first hydrolyzed for 10, 30, 60, 90, or 120 min using a protease M enzyme, followed by subcritical water extraction at 120 °C for 20 min. A control sample was also prepared by suspending white soy flakes in distilled water at pH 9.0 and precipitating the suspension at pH 4.5. The solubility of soy proteins was then evaluated. Results indicated that the protein solubility was slightly improved for the proteins treated by subcritical water, when compared to the control.

In another work, Wang et al. (2019) have treated a soy protein isolate (1–4 w% in phosphate buffer (5 mM, pH 7.0)) at 90 °C by conventional heat treatment and at 120°C by subcritical water treatment. Foaming properties of proteins were then evaluated. Results indicated that the foaming capacity of the proteins was not affected by the conventional heat treatment at 90 °C, when compared to non-heated proteins, but the proteins treated by subcritical water showed a higher foaming capacity. Similar results were observed for the foam volume, with an improvement for the treatment at 120 °C but no effect for the treatment at 90 °C when compared to the native proteins.

Zhang et al. (2015) have processed soy protein isolate with subcritical water at 120, 160, and 200 °C for 20 min. The effect of the subcritical water treatment on the solubility, EAI and ESI, and foaming properties of the soy proteins was investigated. Results indicated that the solubility of the soy proteins was improved by the subcritical water treatments, in comparison with the solubility of the native proteins (control), which was about 22.25%. The proteins treated at 120 °C showed the highest solubility (~36.25%), followed by the ones treated at 200 °C (~35.00%) and at 160 °C (~31.25%). Emulsifying activity index was also improved by the subcritical water treatment for all temperatures, while the ESI was improved for the proteins treated at 160 and 200 °C. A similar pattern was observed for the foaming capacity and for the foaming stability as for the ESI, that is to say that the proteins treated at 160 and 200 °C showed an improvement when compared to the control, but not the proteins treated at 120 °C.

The impact of subcritical water treatment on the functional properties of zein proteins was also studied by Zhang et al. (2018). The results showed that subcritical water treatment, with different times (20–120 min) and temperatures (110–170 °C) increased the solubility, foam capacity, and foam stability of zein protein. For the solubility, the temperature of 110 °C showed the highest value (~37%), while for the foam capacity and foam stability, the highest values were observed at 130 °C (25.6% ± 0.8% and 51.3% ± 1.3%, respectively).

5.5 Conclusion and Future Perspectives

As aforementioned, the interest in plant proteins is forecast to grow significantly in the upcoming years due to the growing trend of veganism, flexitarian, and vegetarian diets, and manufacturer involvement in plant-based proteins driven by strong retail sales in plant-based products. This is reflected in the large number of scientific papers that have recently been published on the extraction of plant proteins using emerging technologies. Among these technologies there are emerging green solvent extraction technologies, in particular ATPE, DES extraction, and subcritical water extraction, that show promising potential for plant protein extraction. Aqueous two-phase extraction and DES extraction are considered green extraction technologies. Aqueous two-phase systems usually consist of components that are GRAS and, in general, both phases contain more than 80% water. Deep eutectic solvents are easy to synthesize and have low volatility and high dissolution power and high biodegradability. Both technologies have great potential for plant protein extraction, as illustrated in this chapter. Concerning subcritical water extraction, it is also environmentally friendly since it uses hot water (100 to 374 °C) in a liquid state under high pressure (up to 22 MPa) to extract the proteins. However, under those conditions, subcritical water may hydrolyze the proteins and can thus be considered as a good alternative to enzymatic hydrolysis for the production of peptides and amino acids, since it is less expensive and faster. All three technologies are also easily scalable.

However, more research works are needed to assess the full potential of these technologies for plant protein extraction. So far, a limited number of plant sources have been considered. There is a need to optimize the application of each process for the extraction of plant proteins from oilseeds, pulses, and cereals, and to compare their performances with those of conventional process such as the alkaline extraction-isoelectric precipitation process. More studies are also needed to fully assess the impact of ATPE, DES extraction, and subcritical water extraction on the functional properties and on the protein quality of the extracted proteins. At the moment of writing this book chapter, no study regarding the impact of these three extraction technologies on the protein quality can be found in the scientific literature.

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Enzyme-Assisted Extraction of Plant Proteins

6

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Abstract

Enzyme-assisted extraction (EAE) is an environmentally friendly green processing technique used to aid protein extraction from different plant sources. This is due to its mild operating conditions, reduced waste generation and low energy consumption compared to chemical and physical extraction approaches. A range of food grade carbohydrase and protease preparations have been employed to aid protein extraction/solubilisation from different plant sources by hydrolysis of the plant cell wall and the proteins therein. Different statistical tools can be employed to optimise enzyme treatment parameters including enzyme:substrate, pH, incubation temperature and hydrolysis duration to yield maximal protein recovery. While EAE facilitates protein recovery, it may also enhance the nutritional (digestibility) and techno- and bio-functional properties of the extracted proteins, particularly when using protease-assisted extraction. Combining EAE with physical techniques, e.g., ultrasonic processing, for biomass pre-treatment can enhance plant cell wall disruption with a view to enhancing protein extraction efficiency. This approach can facilitate economic feasibility by reducing the energy required and the quantity of enzyme used and, therefore, the overall cost of the extraction process. An overview of the application of EAE in protein/peptide recovery from different plant sources including oilseeds, nuts, cereals, pulses and algae is provided herein.

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Keywords

Enzyme-assisted extraction · Proteases · Carbohydrases · Plant proteins · Hydrolysis conditions

6.1 Introduction

The demand for plant protein ingredients is growing rapidly due to the scarcity of resources and the environmental impact associated with the increased production of animal-derived proteins to meet the ever-increasing global protein need. The growing consumer demand for plant-based proteins is driven by various factors including increased awareness of personal well-being as well as an increased focus on environmental and ethical concerns (Lonnie and Johnstone 2020). Food and agricultural by-products generated during harvesting, post-harvesting and processing represent a valuable source of low-cost protein which if appropriately utilised can help in reducing food waste and the environmental impact of food production (Contreras et al. 2019; Görgüç et al. 2020; Kamal et al. 2021). Therefore, proteins derived from different plant sources such as legumes, oilseeds, nuts, cereal, algae and their processing by-products are increasingly being exploited with a view to reducing and/or substituting for animal-derived proteins. The employment of appropriate extraction technologies is considered as a key step in plant protein bio-refinery in order to achieve high extraction efficiency and maximal utilisation of limited natural resources.

Plant proteins are present in different forms and locations within plant cells. These include structural proteins in the cell wall, and storage and functional proteins in the cytoplasm and organelles in the form of enzymes and complexes bound to other compounds, e.g., carbohydrates, lipids and pigments. Compared to animal cells, plant cells contain an extra cell wall barrier which is a highly complex structure generally composed of molecules such as cellulose, hemicellulose (xyloglucans), pectin and glycoproteins (Casas and Domínguez González 2017; Nadar et al. 2018). The complexity and rigidity of the plant cell wall represent a challenge during the protein extraction process. A range of extraction technologies can be employed to aid plant protein extraction including the aqueous and pH-shift methods (using acid and alkaline) and novel techniques such as high pressure, ultrasound, microwave, pulse-electric field, sub/super critical water extraction, etc., along with enzyme-assisted extraction (EAE).

EAE techniques are considered as rapid, mild, non-toxic and more environmentally friendly with relatively less chemical waste generated when compared to chemical and physical protein extraction approaches (Cheng et al. 2019). For example, conventional protein extraction protocols generally involve long extraction times in aqueous solution, while enzyme-assisted optimised extraction can be performed in a shorter time while yielding similar or higher protein yields (Nikbakht Nasrabadi et al. 2021). Harsh extraction conditions, e.g., at alkaline and acidic pHs, and the heat generated from mechanical processing can lead to denaturation of

proteins resulting in diminished technofunctional properties, particularly in the solubility of the extracted proteins. These processes may also negatively impact the nutritional status of the extracted proteins through amino acid racemisation from L- to non-metabolisable D-forms and the formation of lysinoalanine (Sari et al. 2015). Furthermore, the use of chemical agents during the extraction process may result in the generation of large volumes of waste which has associated environmental impacts due to the need for treatment and disposal of same. Therefore, the application of EAE is a promising "greener" approach to aid in the process of plant protein extraction.

This chapter provides a general overview of EAE and the operating conditions for same which impact protein extraction efficiency and protein yield. The application of EAE in aiding protein recovery, from different plant protein sources, in comparison with other non-enzyme assisted protein extraction techniques is also described herein.

6.2 The EAE Process

Enzyme-based techniques can, in the first instance, enable protein extraction from plants by degrading the complex carbohydrate-rich plant cell wall. The plant cell wall contains oligo-/poly-saccharides and glycoproteins, the structures of which can vary depending on the plant species. Consequently, food-grade carbohydrase preparations need to be carefully chosen on the basis of knowledge of the cell wall components of the specific plant species of interest. EAE processes involve hydrolysis of the plant cell wall using a specific carbohydrase or a combination of carbohydrases. Furthermore, in certain instances, EAE may involve hydrolysis processes using a combination of carbohydrases and proteases.

The EAE approach is also associated with beneficial outcomes in relation to technofunctional property enhancement such as improved solubility, gelling and emulsifying properties, and water and oil binding capacity of the extracted proteins/peptides (Nikbakht Nasrabadi et al. 2021). In addition to the advantages already described, there are some challenges associated with the application of EAE approaches for the recovery of plant proteins. One such challenge may be related with the need to inactivate/remove the enzyme activity from the extracted proteinaceous ingredient in order to minimise further enzyme action when the protein ingredient is incorporated into formulated foods containing multiple other ingredients. Carry-over of the enzyme activity arising from the EAE process may have implications for the shelf-life of the ingredient itself and formulated foods containing the ingredient. Furthermore, using enzymes adds an additional cost to the extraction process which may not be cost effective when compared to conventional protein extraction protocols. Therefore, as already outlined, careful selection of specific enzymes for each plant source is essential as the constituent cell wall and intracellular polysaccharides/proteins vary significantly across different plant species. Furthermore, crude enzyme preparations may contain side activities which may modify the characteristics of the extracted proteins and there may be inconsistencies

Table 6.1 Advantages and limitations of enzyme-assisted extraction of plant proteins compared to other approaches

Advantage	Limitation
• Milder conditions and straightforward process • Less chemical waste • Lower energy consumption • High protein yield within shorter time • Wide range of food-grade enzyme preparations available • Possibility to use highly specific enzyme activities • Amenable to scale up	• Cost of the enzyme • Requires details of cell wall composition of each plant protein starting material to aid selection of the most appropriate enzyme activity • Requires an optimisation process to minimise enzyme dosage to achieve high recovery yield • May require an additional step during enzyme inactivation or separation • Variation in enzyme activity between batches which may contain some side activities

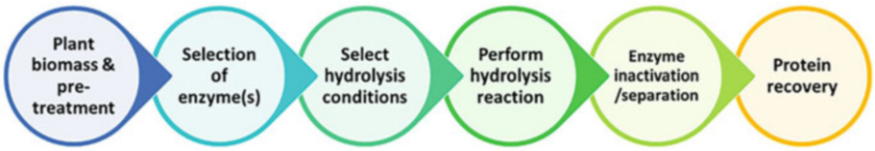

Fig. 6.1 Schematic overview of enzymatic-based extraction of plant protein

in terms of enzyme activities between batches (Nadar et al. 2018; Cheng et al. 2019). Some of the advantages and limitations of employing EAE are outlined in Table 6.1.

The enzyme-assisted plant protein extraction process involves the selection of enzyme preparation(s), optimisation of hydrolysis conditions and performance of the hydrolysis reaction under selected parameters, termination of the reaction by inactivation of the enzyme (or by its separation) and finally recovery of the proteinaceous fractions (Fig. 6.1). Protein recovery following EAE can generally be achieved using processes such as isoelectric precipitation, salting out (e.g. using ammonium sulfate) and membrane processing. The hydrolysis process itself can be carried out using a single enzyme preparation or a combination of more than one enzyme preparation. Furthermore, the EAE process may be operated as a single batch or as a continuous process using an enzyme membrane bioreactor. The latter approach provides advantages over a batch operation as it facilitates enzyme recovery and enrichment of the proteinaceous fraction(s) in a single operation (Cheng et al. 2019; Kleekayai and FitzGerald 2021). Recycling of the enzyme(s) used is recognised as a key advantage helping to minimise the cost and make the process more feasible for commercial scale applications (Nadar et al. 2018; Cheng et al. 2019).

Enzyme-assisted processes can be optimised to achieve maximum protein recovery while minimising the quantity of enzyme required. Statistical tools, such as design of experiments (DOE), factorial design, Box–Behnken Design (BBD), central composite design (CCD) and response surface methodology (RSM), have been employed for optimisation of the extraction process, as outlined in Table 6.2. Several parameters can be studied, e.g., enzyme to substrate ratio (E:S), reaction pH,

Table 6.2 Summary of some recent studies employing statistical tools for optimisation of enzyme-assisted protein extraction from plant sources

Plant biomass	Enzyme	Tool	Optimised conditions	Protein yield (% (w/w))	Protein purity (% (w/w))	Reference
Sugar beet leaves	Pectinex® ultra SP-L	RSM	Aqueous:biomass 27.65 mL/g, E:S 8% (v/w), 54.25 °C, 81.35 min	79.01	69.08	(Akyüz and Ersus 2021)
Defatted *Moringa oleifera* leaves	Viscozyme® L	CCD (fractional factorial 2^{5-1}), RSM	Flour:aqueous 1:20 (w/v), E:S 60 FGU/mL, pH 5.5, 30 °C, 30 min	14.20	55.70	(Benhammouche et al. 2021)
Dried milled nori (*Pyropia yezoensis*)	Agarase (from *Pseudomonas atlantica*) & Cellulase (from *Trichoderma spp.*)	BBD (3^3), RSM	Biomass:aqueous 1/26 (w/v), agarase 104 U/g + cellulase 100 U/g, 29 °C, 5 h	6.953 mg R-PE/g dw	0.287 (A_{565}/A_{280})	(Wang et al. 2020)
Air dried milled brown seaweed (*Macrocystis pyrifera*)	Cellic® CTec3	CCD, RSM	E:S 1/10 (v/w), pH 4.5, 50 °C, 18 h	74.60	NA	(Vásquez et al. 2019)
Air dried milled red seaweed (*Chondracanthus chamissoi*)	Cellic® CTec3	CCD, RSM	E:S 1/10 (v/w), pH 4.5, 50 °C, 12 h	36.10	NA	(Vásquez et al. 2019)
White sorghum flour (variety Perla 101)	AMG® 300 L	Factorial design (3^3), RSM	20% (w/v) flour in 0.5 M sodium acetate buffer pH 4.5, E:S 0.086% (v/w), 83 h	50.80	49.16	(Castro-Jácome et al. 2020)
Sesame bran	Alcalase® 2.4 L	CCD	Biomass:aqueous 1:10, E:S 1.94 AU/100 g, 49 °C, 98 min, combined with microwave treatment (750 W)	91.70	NA	(Görgüç et al. 2019b)

(continued)

Table 6.2 (continued)

Plant biomass	Enzyme	Tool	Optimised conditions	Protein yield (% (w/w))	Protein purity (% (w/w))	Reference
Sesame bran	Alcalase® 2.4 L	CCD, RSM	E:S 1.248 AU/100 g, pH 9.8, 51 °C, 68 min	79.30	NA	(Görgüç et al. 2019a)
Sesame bran	Viscozyme® L	CCD, RSM	E:S 69.6 FBG/100 g, pH 6.0, 51 °C, 117 min	41.70	NA	(Görgüç et al. 2019a)
Defatted okara (soy pulp) flour	Viscozyme® L	CCRD (2^3), RSM	E:S 4%, pH 6.2, 53 °C, 2 h	29.80	56.00	(de Figueiredo et al. 2018)
Freeze-dried red seaweed *(Mastocarpus stellatus)*	Endo-1,4-β-xylanase	CCD, RSM	Biomass:50 mM phosphate buffer 1:100 pH 6.45, E:S 13.18 mg xylanase/g dw, 12 °C, 6 h in the dark	1.99 mg R-PE/g dw	0.36 (A_{565}/A_{280})	(Nguyen et al. 2017)
Defatted sacha inchi (*Plukenetia volubilis*) kernel cake	Alcalase® 2.4 L	CCD, RSM	Biomass:aqueous 1:50, pH 9.0, E:S 5.6%, 50 °C, 40.4 min	44.70	NA	(Chirinos et al. 2017)

RSM response surface methodology, *CCD* central composite design, *BBD* Box–Behnken Design, *CCRD* central composite rotatable design, *E:S* enzyme: substrate, *AU* Anson unit, *FBG* fungal β-glucanase unit, *R-PE* R-phycoerythrin, *NA* not available

incubation temperature and time (Fig. 6.2). These factors influence enzyme activity and, therefore, can impact protein yield. The application of statistical tools can determine the effects of an individual factor and the interaction of different factors on the response of interest, i.e., protein yield. The impact of different processing variables (factors) can be determined at small/lab-scale using a series of experiments dictated by the DOE. The resultant data can then be assessed using RSM to predict the optimum processing conditions (FitzGerald et al. 2020). The predicted optimum conditions need to be experimentally tested and the values obtained can be compared to the predicted values obtained from the models. In certain instances, this may require some additional experimentation in order to identify the optimal processing conditions. However, the advantage of this approach is that it reduces the number of optimisation experiments required to a minimum and thus saves on time and cost. Table 6.2 provides an overview of some recent studies reporting optimised enzyme-assisted plant protein extractions using different statistical tools.

6.3 Factors Affecting EAE Protein Yield

As outlined previously, a range of parameters need to be examined when optimising protein extraction and recovery from different plant sources using EAE approaches. These include: the choice and concentration of enzyme(s) used, the biomass concentration and particle size, biomass pre-treatment, and the reaction pH, temperature and duration (Fig. 6.2).

6.3.1 Choice of Enzyme(s)

Due to differences in the specific cell wall components and structures of each plant species, judicious choice of enzyme plays a major role in protein yield. In general, two groups of enzymes have been employed to aid protein extraction, i.e., carbohydrases and proteases. Table 6.3 outlines some commercially available food-grade enzyme preparations commonly used for enzyme-assisted plant protein extraction, their optimum conditions and their mode of action. Carbohydrases hydrolyse polysaccharides to oligo- and mono-saccharides. Once the structural compounds in the cell walls are disrupted, intracellular proteinaceous components may be released, as illustrated in Fig. 6.2. The application of proteases can enhance protein yield by detaching proteins from polysaccharide matrices and by further degrading large protein molecules to smaller molecular mass proteins and peptides. This in turn promotes the solubilisation of the proteinaceous components (Liu et al. 2016; Casas and Domínguez González 2017; Nadar et al. 2018; Kumar et al. 2021).

Specific carbohydrase groups, e.g., cellulases, hemicellulases, xylanases and pectinases, are used for protein extraction depending on the cell wall composition of the plant species. Due to the diverse composition of the plant cell wall, carbohydrase enzyme cocktails are often used. The carbohydrases employed during EAE generally have mildly acidic pH optima (pH 4–6) and are mainly derived from

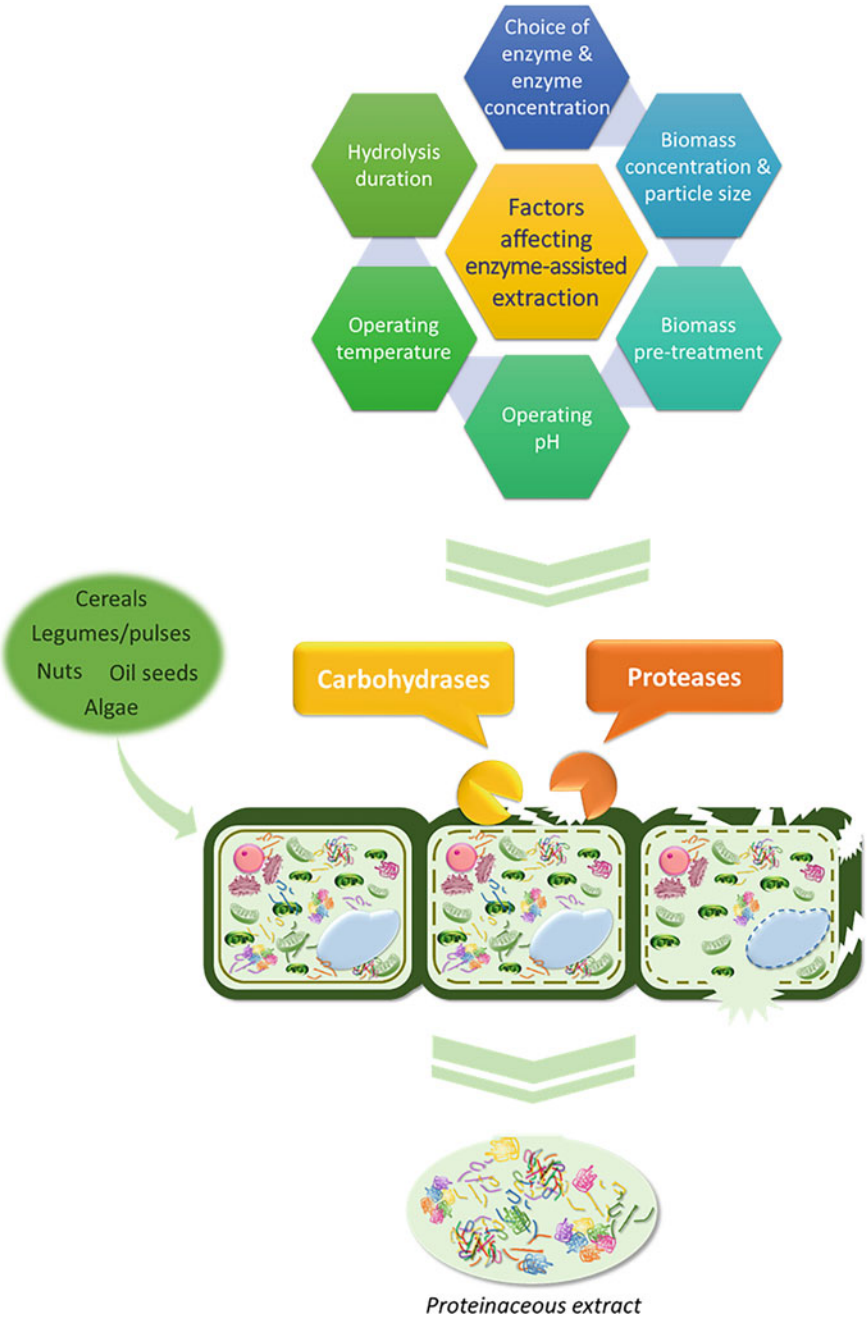

Fig. 6.2 Enzyme-assisted protein extraction and the associated factors affecting protein yield (modified from Kumar et al. (2021))

Table 6.3 Examples of enzyme preparations commonly used for protein extraction from plant sources (modified from Nadar et al. (2018), Habeebullah et al. (2021))

Enzyme	Source	Optimum conditions		Enzyme specificities	Enzyme units[a]	Supplier
		pH	Temperature (°C)			
Carbohydrase						
AMG® 300 L	*Aspergillus niger*	4.5	60	Exo-1,4-α-D-glucosidase	300 AGU/mL	Novozymes
Celluclast® 1.5 L FG	*Trichoderma reesei ATCC 26921*	4.5	50	Cellulase (endo-(1,4)-β-D-glucanases, exo-(1,4)-β-D-glucanases and β-glucosidases)	700 EGU/g	Novozymes
Cellic® CTec3	*Trichoderma reesei*	4.5	50	Endo- and exo-cellobiohydrolases, β-glucosidase, hemicellulases	1700 BHU/g	Novozymes
Termamyl® 120 L	*Bacillus licheniformis*	6	60	Heat-stable α-amylase	120 KNU/g	Novozymes
Ultraflo™ L	*Humicola insolens*	7	60	Heat-stable multi-active endo-1,4-β-glucanase, endo-1,4-β-xylanase and some side activities of cellulase, hemicellulase, pentosanase	45 FBG/g	Novozymes
Viscozyme® L	*Aspergillus aculeatus*	4.5	50	Arabinase, cellulase, β-glucanase, hemicellulose, xylanase	100 FBG/g	Novozymes
Pectinex® ultra SP-L	*Aspergillus aculeatus*	4.5	60	Polygalacturonase, pectinesterase, pectin trans-eliminase, hemicellulase, cellulase	3800 PGNU/g	Novozymes
Protease						
Alcalase® 2.4 L	*Bacillus licheniformis*	8	50	Serine endo-peptidase (subtilisin A)	2.4 AU/g	Novozymes
Flavorzyme® 500MG	*Aspergillus oryzae*	7	50	Endo- and exo-peptidase complex	500 LAPU/g	Novozymes
Neutrase® 0.8 L	*Bacillus amyloliquefaciens*	6	50	Metalloprotease	0.8 AU/g	Novozymes
Protamex®	*Bacillus* sp.	6	40	Alkaline endoprotease complex	1.5 AU/g	Novozymes

(continued)

Table 6.3 (continued)

Enzyme	Source	Optimum conditions		Enzyme specificities	Enzyme units[a]	Supplier
		pH	Temperature (°C)			
Umamizyme™	*Aspergillus oryzae*	7	50	Neutral endo- and exo-peptidase complex	70 LGGU/g	Amano
Promod® 144 MG	Papaya *(Carica papaya)* latex	7	65	Cysteine proteinase	100 TU/mg	Biocatalysts

[a] *AGU* amyloglucosidase unit, *EGU* endoglucanase unit, *BHU* biomass hydrolysis unit, *KNU* kilo Novo Units α-amylase, *FBG* fungal β-glucanase unit, *PGNU* polygalacturonic units, *AU* Anson unit, *LAPU* leucine aminopeptidase unit, *LGGU* Leucylglycylglycine unit, *TU* tyrosine unit

microbial sources (Table 6.3). However, differences in the specificity and activity of the same carbohydrase group derived from different microbial sources can have a major influence on protein recovery. For instance, α-amylase from *Bacillus licheniformis* was shown to be more effective in the removal of starch from potato pulp giving an increase in protein recovery from 75 to 100% (w/w) compared to α-amylase from other *Bacillus* sp. (Waglay et al. 2016). The application of carbohydrases can also contribute to reducing the level of non-proteinaceous components in the protein extract. This can contribute to enhancing the purity of the resultant protein extract. For instance, the application of carbohydrases such as Viscozyme® and Celluclast® has been reported to reduce the level of phenolic compounds in the protein extract obtained from Pacific dulse (*Devaleraea mollis*) (Mendez and Kwon 2021).

Proteases can be classified into 2 main groups based on the location of their cleavage of the protein molecule, i.e., they can cleave internally or at the terminal positions in the polypeptide chain, and these are known as endo-proteinases and exo-peptidases, respectively. Many proteases work under slightly alkaline conditions having optimum pH and temperature values between pH 8–10 and 45–60 °C, respectively (Sari et al. 2015). Similarly, to the choice of carbohydrases, proteases with broad specificity are often selected during the EAE of plant proteins. This lack of specificity brings about effective breakdown of cell wall structural proteins along with intracellularly located storage protein complexes. It has been reported that the application of proteases may beneficially prevent the released proteins from forming complexes with other components such as carbohydrates and phytates under specific extraction conditions (Kumar et al. 2021). In addition, the solubilised proteins and peptides obtained from the hydrolysis process have been reported to possess numerous potential health promoting effects such as antihypertensive, hypocholesterolaemic, anticarcinogenic, immunomodulatory and opioid activities (Görgüç et al. 2020).

Some studies suggest that the use of proteases is more effective in aiding protein extraction from plant biomass compared to carbohydrases. However, this can be species-dependent. For instance, at an E:S of 0.10% (w/v) the proteases preparations Flavourzyme® 500MG, Alcalase® 2.4 L FG and Neutrase® 0.8 L gave higher protein yields from macroalgae (*Fucus serratus*, *Ascophyllum nodosum* and *Polysiphonia fucoides*), compared to the use of the carbohydrases preparations Viscozyme® L, AMG® 300 L, Cellucast® 1.5 L FG, Termamyl® 120 L and Ultraflo™ L at similar E:S values (Habeebullah et al. 2021). However, in the same study, it was reported that hydrolysis of *Fucus vesiculosus* with the carbohydrase Termamyl® 120 L led to the highest protein yield. In the case of sesame bran, the application of the protease preparation Alcalase® yielded a higher protein recovery, i.e., 79.30% (w/w) compared to the application of Viscozyme® L which gave a protein recovery of 41.70% (w/w) (Görgüç et al. 2019a).

Therefore, a combination of proteases and carbohydrases is also employed during enzyme-assisted plant protein extraction with a view to increasing protein yield from a range of plant sources, e.g., rapeseed, chickpea, brewers' spent grain (BSG) and *Palmaria palmata*, as outlined in Table 6.4. For the red seaweed *P. palmata,*

hydrolysis using either Celluclast® or Shearzyme® in combination with Alcalase® led to a yield of 90.20% (w/w), while when a single enzyme was employed the protein yield was <55.20% (w/w) (Naseri et al. 2020). The protein yield from screw pressed white clover (*Trifolium repens*) and ryegrass (*Lolium perenne*) increased by up to 19.00 and 25.00% (w/w), respectively, when a combination of carbohydrases (Cellic® CTec2 and Cellic® HTec2) and a protease (Savinase 16.0 L) was used, compared to the use of protease alone (Dotsenko and Lange 2017). Table 6.4 provides a summary on the application of proteases, carbohydrases and their combinations for plant protein extraction, the hydrolysis conditions used and the protein yield obtained from different plant sources.

6.3.2 Enzyme Concentration

The rate of hydrolysis during EAE is dependent on the enzyme activity within a given carbohydrase or protease preparation. It is well documented that increasing enzyme concentration during EAE generally results in a greater protein yield (Görgüç et al. 2019a, b, Singh et al. 2019, Vásquez et al. 2019, Yu et al. 2019, Castro-Jácome et al. 2020). Yu et al. (2019) reported a significant increase in protein yield, i.e., from 34.00 to 61.60% (w/w), when the enzyme concentration was increased during protein extraction from BSG from 1 to 20 μL Alcalase®/g BSG. Similar results were observed for protein extraction from brown (*Macrocystis pyrifera*) and red (*Chondracanthus chamissoi*) seaweeds using Cellic® CTec3 when the E:S was increased from 1:100 to 1:10 (v/w) (Vásquez et al. 2019). Statistical analysis revealed that the protein content in the extracts was significantly ($p < 0.05$) influenced by the E:S for both algal species. Another study also found that the enzyme activity had a significant ($p < 0.001$) impact on the protein yield obtained from sesame bran hydrolysis using Viscozyme® L or Alcalase®. An increase in protein yield from 31.00 to 44.80 and 41.10 to 77.70% (w/w) was observed when the enzyme activity was increased from 6.0 to 120.0 fungal β-glucanase (FBG) units/100 g and 0.12–2.40 Anson unit (AU)/100 g for Viscozyme® L and Alcalase®, respectively (Görgüç et al. 2019a, b). Benhammouche et al. (2021) reported that the Viscozyme® concentration used had the highest influence ($p < 0.001$) on protein extraction yield from defatted *Moringa oleifera* leaves when compared to variations in other parameters, i.e., temperature and incubation time. These authors also reported that reaction pH and biomass concentration had no significant effect ($p > 0.05$) on protein yield, in this instance.

Nonetheless, the use of higher enzyme concentrations has implications for the cost of the extraction process and may not always lead to a greater protein yield. For instance, no increase in the concentration of kafirin (a storage protein extracted from white sorghum flour) was observed on increasing the E:S of the carbohydrase AMG® 300 L up to 0.15% (v/w) (Castro-Jácome et al. 2020). This was associated with the release of hydrolysis products including sugars, tannins and polyphenols which inhibited the enzyme. This phenomenon has also been observed when

Table 6.4 Summary of some enzyme-assisted extraction studies for plant proteins or their protein hydrolysates

Plant biomass	Enzyme	Enzyme-assisted extraction conditions	Protein recovery yield (% (w/w))	Protein content (% (w/w))	Reference
Carbohydrase aided extraction					
Defatted flaxseed	Cellulase	E:S 2.00% (w/w), 37 °C, pH 5.0, 4 h	65	30.72	(Tirgar et al. 2017)
Peanut meal	Viscozyme® L	E:S 1.35% (w/w), 52 °C, pH 7.2, 1.5 h	79	24.44	(Liu et al. 2020)
Lentil	Distizym® AG (containing glucoamylase)	170 g/L milled lentil, E:S 1.7 U/g glucoamylase, pH 7, 50 °C, 3 h	36.00	50.00	(Bildstein et al. 2008)
White bean	Distizym® AG (containing glucoamylase)	170 g/L milled white bean, E:S 1.7 U/g glucoamylase, pH 7, 50 °C, 3 h	72.00	49.00	(Bildstein et al. 2008)
Barley	α-amylase, amyloglucosidase and β-1,3,4-glucanase (followed by IEP)	α-amylase 10,000 U/g, 65 °C, pH 6.5, 1 h; amyloglucosidase 660 U/g, 40 °C, pH 6.5, 16 h; β-1,3,4-glucanase 8 U/g, 37 °C, pH 5.0, 1 h	78.30	41.40	(Houde et al. 2018)
Oat bran	Amyloglucosidase	E:S 8 U/g defatted bran, pH 5.5	ND	82.00	(Jodayree et al. 2012)
Oat bran	Viscozyme® L	E:S 3 FBG/g oat bran, 44 °C, pH 4.6, 2.8 h	ND	56.00	(Guan and Yao 2008)
Palmaria palmata	Celluclast® 1.5 L (cellulase) and Shearzyme® 500 L (xylanase)	1:30 (w/v) dried milled algae, E:S 480 U/g dw, pH 5, 40 °C, 24 h, centrifugation, followed by alkaline aided extraction on the pellet	67.00	ND	(Harnedy and FitzGerald 2013)
Palmaria palmata	Celluclast® 1.5 L (cellulase) and Shearzyme® 500 L (xylanase)	1:28 (w/v) dried milled algae, homogenisation, E:S 50 U/g dw, pH 5, 40 °C, 18 h,	69.80–75.60	ND	(Maehre et al. 2014)

(continued)

Table 6.4 (continued)

Plant biomass	Enzyme	Enzyme-assisted extraction conditions	Protein recovery yield (% (w/w))	Protein content (% (w/w))	Reference
		centrifugation, followed by alkaline aided extraction on the pellet			
Devaleraea mollis	Celluclast® 1.5 L (Cellulase) Viscozyme® L (polysaccharidases including arabinose, cellulase, B-glucanase, hemicellulose and xylanase)	1:15–1:30 (w/v) dried milled algae, E:S 2% (v/v), pH 5–6, 50 °C, 24 h, centrifugation, followed by sequential water-, saline-, alkaline- and ethanol-soluble protein extraction approach on the pellet	58.80–80.17	ND	(Mendez and Kwon 2021)
Macrocystis pyrifera	Cellic® CTec3 (cellulase, hemicellulose and β-glucosidase)	1:50 (w/v) dried milled algae, E:S 0.1% (v/w) dw, pH 4.5, 50 °C, 18 h, centrifugation and acetone precipitation of protein in the supernatant	74.60	ND	(Vásquez et al. 2019)
Chondracanthus chamissoi	Cellic® CTec3 (cellulase, hemicellulose and β-glucosidase)	1:50 (w/v) dried milled algae, E:S 0.1% (v/w) dw, pH 4.5, 50 °C, 12 h, centrifugation and acetone precipitation of protein in the supernatant	36.10	ND	(Vásquez et al. 2019)
Spirulina platensis	Cellulase	1:10 (w/v) dried milled algae, E:S 1.0% (w/w) protein, pH 5, 50 °C, 3 h, centrifugation	82.00	ND	(Mahali and Sibi 2019)

Chlorella sp. *Chlamydomonas* sp., *Scenedesmus* sp., Mixed culture (M.C. sp.)	Lysozyme	1:20 (w/v) wet algae, E:S 0.33% (w/w) dw, pH 7, 37 °C, 16 h, centrifugation	79.00–97.00	ND	(Al-Zuhair et al. 2017)
Protease aided extraction					
Sesame	Protex 7 L	E:S 2.0% (w/w), 45 °C, pH 7, 2 h	87.10	3.5–5.9	(Latif et al. 2008)
Palm kernel	Trypsin	E:S 1.40% (w/w), 37 °C, pH 9.5, 6 h	72	14–20	(Chee et al. 2012)
Almond cake	FoodPro® alkaline protease	E:S 0.85% (v/w), 50 °C, pH 9.0, 2 h	75	37	(Souza et al. 2019)
Dehulled peanut	Alcalase	E:S 1.5% (w/w), 60 °C, pH 9.5, 5 h	88.21	22.35	(Jiang et al. 2010)
Lentil	Alcalase® 2.4 L or bromelain	15 mg/mL lentil protein isolate, E:S 1% (w/v) Alcalase (3.030 U/mL or bromelain (100–1500 GDU U/g), pH 7.5, RT, 15 min	23.00–70.00	20.00–60.00 (determined using the Bradford assay)	(Xu et al. 2021)
Chickpea	Alcalase® 2.4 L	1 g/mL chickpea protein isolate, E:S 1 U/mg protein, pH 8.0, 50 °C, 210 min	ND	79.21–83.75	(Ghribi et al. 2015)
	Alcalase® 2.4 L	0.1 g/mL chickpea albumin or globulins, E:S 0.3 U/g protein, pH 8.0, 50 °C, 90 min	ND	75.60–97.90 (determined using the BCA assay)	(Quintero-Soto et al. 2021)
	Alcalase® 2.4 L or bromelain	1.5% TS of chickpea protein isolate, E:S 1% (w/v) Alcalase®, pH 7.5, RT, 15 min or E:S 1% (w/v) bromelain, pH 6.5, RT, 15 min	23.00–64.00	18.00–50.00 (determined using the Bradford assay)	(Xu et al. 2021)

(continued)

Table 6.4 (continued)

Plant biomass	Enzyme	Enzyme-assisted extraction conditions	Protein recovery yield (% (w/w))	Protein content (% (w/w))	Reference
	Alcalase® 2.4 L	5% (w/v) chickpea protein concentrate, E:S 1% (v/v), pH 7.0, 50 °C, 210 min	ND	69.10–68.70	(Felix et al. 2020)
	Alcalase® 2.4 L and/or Flavourzyme	Chickpea protein isolate, E:S 2% (v/w) Alcalase (0.4 AU/g protein), pH 8, 50 °C, E:S 2% (v/w) Flavourzyme (100 LAPU/g protein), pH 7, 50 °C and combination of both enzymes at E:S 2%	ND	91.20–91.80	(Clemente et al. 1999)
Chickpea by-products (non-conforming chickpeas, skins and plant parts)	Alcalase® 2.4 L, papain, pepsin or trypsin	0.2 g/mL ground by-product in 10 mM phosphate buffer for Alcalase, trypsin, papain and the combination of Alcalase and papain E:S 1% (v/w) Alcalase, pH 6.5–8.5, 60 °C, 2 h E:S 1% (w/w) trypsin, pH 7.0–9.0, 37 °C, 2 h E:S 1% (w/w) papain, pH 6–7, 65 °C, 2 h E:S 1% (w/w) papain and 1% (v/w) Alcalase, pH 6.5–7, 62.5 °C, 2 h 0.2 g/mL ground by-product in 10 mM HCl, E:S 1% (w/w) pepsin, pH 2–4, 37 °C, 2 h	23.00–43.00	35.00–39.00	(Prandi et al. 2021)

Pigeon pea (*Cajanus cajan*) protein isolate	Alcalase® 2.4 L or bromelain	Biomass: aqueous 15 mg/mL, E:S 1% (w/v) Alcalase (3.030 U/mL), pH 7.5, RT, 15 min or E:S 1% (w/v) bromelain (100–1500 GDU / g), pH 6.5, RT, 15 min	23.00–92.00	20.00–80.00 (determined using the Bradford assay)	(Xu et al. 2021)
	Alcalase® 2.4 L, pancreatin or pepsin and pancreatin	E:S 1% Alcalase, pH 8.0, 50 °C, 4 h, pH stat or E:S 1% pancreatin, pH 7.5, 37 °C, 4 h, pH stat or E:S 1% pepsin + pancreatin, pH 2.0, 37 °C and pH 7.5, 37 °C, pH stat; fractionation of hydrolysates using 1, 3, 5 and 10 kDa membranes	4.20–53.88	23.90–56.80	(Olagunju et al. 2018)
Pea	Alcalase® 2.4 L	5% (w/v) protein isolate, E:S 4% (v/w), pH 9, 50 °C, 6 h, pH stat; ultrafiltration 1 kDa cut-off membrane and SPE fractionation with 0.5, 1, 1.5, 2 and 5% NH_4OH	0.42–38.17	22.37–100.00	(Li and Aluko 2010)
	Molsin (acid protease from *Aspergillus saitoi*)	4% (w/v) pea flour, E:S 1:10, pH 2.8, 40 °C, 90 min followed by centrifugation or without	ND	19.90–22.40	(Periago et al. 1998)
Pea by-products (non-conforming peas, skins and plant parts)	Alcalase® 2.L, papain, pepsin, trypsin or combination of Alcalase® 2.4 L and papain	0.2 g/mL ground by-product in 10 mM phosphate buffer for Alcalase, trypsin, papain and the combination of Alcalase and papain E:S 1% (v/w) Alcalase,	12.00–58.00	37.00–66.00	(Prandi et al. 2021)

(continued)

Table 6.4 (continued)

Plant biomass	Enzyme	Enzyme-assisted extraction conditions	Protein recovery yield (% (w/w))	Protein content (% (w/w))	Reference
		pH 6.5–8.5, 60 °C, 2 h E:S 1% (w/w) trypsin, pH 7.0–9.0, 37 °C, 2 h E:S 1% (w/w) papain, pH 6–7, 65 °C, 2 h E:S 1% (w/w) papain and 1% (v/w) Alcalase, pH 6.5–7, 62.5 °C, 2 h 0.2 g/mL ground by-product in 10 mM hydrochloric acid, E:S 1% (w/w) pepsin, pH 2–4, 37 °C, 2 h			
Rice bran	Papain	E:S 0.1% (w/w), 37 °C, 1 h, pH 8.0	54.00	89.80	(Bandyopadhyay et al. 2012)
Chlorella fusca	Protex 40XL	Biomass:aqueous 1:25 (w/v) protein, E:S 1 and 5% (v/w, protein basis), pH 11, 60 °C, 24 h, centrifugation	49.10–58.00	ND	(Sari et al. 2016)
Chlorella fusca meal	Protex 40XL	Biomass:aqueous 1:25 (w/v) protein, E:S 1 and 5% (v/w, protein basis), pH 11, 60 °C, 24 h, centrifugation	64.00–73.20	ND	(Sari et al. 2016)
Nannochloropsis gaditana	Alcalase® 2.4 L	Biomass:aqueous 1:10 (w/v) wet weight, E:S 5% (v/w, dw), pH 8, 50 °C, 4 h, centrifugation	35.00	ND	(Safi et al. 2017)

Protease and carbohydrase aided extraction					
Rapeseed cake	Viscozyme® L and Alcalase® 2.4 L	E:S 0.5% (v/w) Viscozyme® L, pH 4.5, 40 min, then adding E:S 0.5% (v/w) Alcalase® 2.4 L, pH 8.0, 40 min	82	ND	(Niu et al. 2012)
Chickpea	Alcalase® 2.4 L and Thermozyme® L340 (containing α-amylase)	12% (w/v) unextruded or extruded chickpea flour (25 mL), 200 μL Alcalase, 60 °C, 30 min and 35 μL Thermozyme® L340, pH 3, 90 °C, 20 min	Unextruded 10.10% total soluble solids Extruded 15.10–21.90% total soluble solids Unextruded with hydrolysis 29.70% total soluble solids Extruded with hydrolysis 56.10–62.00% total soluble solids	9.50–70.00	(Silvestre-de-León et al. 2020)
	Alcalase® 2.4 L and Thermozyme® L340 (containing α-amylase)	Unextruded or extruded 12% (w/v) chickpea flour (1 L), 8 mL Alcalase, 60 °C, 30 min and 1.4 mL Thermozyme®L340, 90 °C, 20 min	29.50–70.10 (TS)	16.30–20.20	(Silvestre-de-León et al. 2021)
Brewers' spent grain	Combination of Shearzyme and Ultraflo Or combination of Alcalase and Flavourzyme	E:S 75 μL/g BSG dw of each enzyme preparation (Shearzyme and UltraFlo), pH 5.0, 50 °C, 4 h or E:S 2% (v/w, Alcalase/g BSG dw), pH 9.0, 50 °C, 2 h and subsequently adding	ND	44.65	(Connolly et al. 2019)

(continued)

Table 6.4 (continued)

Plant biomass	Enzyme	Enzyme-assisted extraction conditions	Protein recovery yield (% (w/w))	Protein content (% (w/w))	Reference
		Flavourzyme E:S 1% (v/w, BSG dw), pH 7.0, 50 °C, 4 h			
Palmaria palmata	Recombinant xylanase, Umamizyme™	1:1 (w/v) wet-milled algae, 60 °C, overnight and centrifugation. 6.67 μg (0.12 U) xylanase/g wet weight 0.67 mg Umamizyme/g wet weight 6.67 μg (0.12 U) xylanase/g wet weight and 0.67 mg Umamizyme/g wet weight	Xylanase: 24.40 (S)/ 62.20 (P) Umamizyme: 63.40 (S)/21.00 (P) Xylanase +Umamizyme: 66.60 (S)/21.00 (P)	ND	(Bjarnadóttir et al. 2018)
Palmaria palmata	Alcalase® 2.4 L and Celluclast® 1.5 L or Shearzyme® 500 L	1:20 (w/v) dried milled algae, 0.2% (v/w) dry weight, pH 4.5, 50 °C, 14 h, centrifugation (P1 + S1), followed by precipitation at pH 3.0 and centrifugation (P2 + S2)	85.5–90.00 (S1) 15.20–20.10 (P2)	ND 37.00–45.90	(Naseri et al. 2020)
Antinutritional factor degrading enzyme					
Rapeseed meal	Phytase	E:S 0.8 U/g, 55 °C, pH 5, 2 h	72.1	38.8	(Rodrigues et al. 2017)

E:S enzyme:substrate, *dw* dry weight, *ND* not determined, *RT* room temperature, *AU* Anson unit, *FBG* fungal β-glucanase unit, *LAPU* leucine aminopeptidase unit, *GDU* gelatin digestion unit, *TS* total solids, *AU* Anson unit, *LAPU* leucine amino peptidase unit, *rpm* revolutions per minute, *BSG* Brewers' spent grain, *SPE* solid-phase extraction, *S* supernatant, *P* pellet

proteases were used, e.g., the protein yield from BSG did not improve with E:S values higher than 20 μL Alcalase® 2.4 L/g BSG (Yu et al. 2019). In the case of white clover or ryegrass protein extraction, the maximum yield was obtained using an E:S of 5 mg Savinase™ 16.0 L/g when an E:S range between 0–20 mg/g dry weight (dw) was investigated (Dotsenko and Lange 2017).

6.3.2.1 Biomass Concentration and Particle Size

The biomass or substrate concentration used during protein extraction determines the total solids (TS) content and enzyme accessibility during the extraction process. Plant biomass concentrations employed during protein extraction are generally in the region of 5–15% (w/v) TS. Working at lower TS would subsequently require further downstream processing, i.e., extract concentration by, e.g., evaporation, which is not a cost-effective approach. On the other hand, working at higher TS (>15%) can negatively impact the rate of hydrolysis. In general, an increase in mass:volume leads to a higher protein yield until an optimum point is reached beyond which no further increase in protein yield is achieved. This was observed in the case of Pectinex Ultra SP-L™ assisted protein extraction from sugar beet leaves where no significantly increase in protein yield was observed when the mass:aqueous reached 1:27.65 g/mL (Akyüz and Ersus 2021). In addition, no significantly increase in protein yield was observed when the TS was greater than 10% (w/v) during pectinase-assisted protein extraction from defatted rapeseed cake (Rommi et al. 2015).

The particle size of the plant biomass is another factor that can affect the extraction yield. In general, a smaller particle size leads to higher extraction yield due to increased surface accessibility by the enzyme(s) (Preece et al. 2017). It also enhances the diffusion of intracellular components into the surrounding aqueous medium (Saad et al. 2019). Grinding of plant biomass and then passage through a small pore sized sieving mesh, e.g., <250 μm or 1 mm for flour and leaf biomass, respectively, is routinely employed to obtain a homogeneous small particle sized material for subsequent extraction. Vishwanathan et al. (2011) reported maximum protein recoveries of 97 and 93% (w/w) from a fine fraction with a particle size <75 μm for soybean flour and okara flour, respectively. Drying the plant biomass prior to extraction has been shown to have a significant impact on the protein yield obtained during subsequent EAE. Dry algal biomass (*Mastocarpus stellatus*) resulted in a higher protein yield (2.94 mg protein/g dw) compared to when wet biomass was used (1.72 mg protein/g dw) (Nguyen et al. 2017). The higher protein yield from the freeze-dried sample may be linked to the smaller particle sizes obtained following homogenisation in liquid nitrogen, whereas the wet biomass was cut into <1 cm pieces prior to freezing. This represents a challenge associated with wet/fresh plant materials, particularly wet plant leaf biomass which requires grinding to increase the surface area prior to extraction. Therefore, dried biomass is more extensively used for protein extraction. However, the choice of the drying process, e.g., freeze-drying vs air drying, can have a major impact on the costs associated with the protein extraction process in addition to potentially impacting the overall quality of the plant proteins.

6.3.2.2 Biomass Pre-Treatment

Physical pre-treatments of plant biomass can contribute to a greater protein extraction yield. Pre-treatment can facilitate cell wall disruption, accessibility to the extraction medium/solvent and increased susceptibility of the plant biomass to the enzyme(s) (Casas and Domínguez González 2017). In addition, combining EAE with other innovative techniques can provide potential benefits such as a reduction in the quantity of enzyme(s) required, shorter incubation times and reduced energy consumption. These can contribute to the economic feasibility of the protein extraction process. A range of pre-treatment techniques have been employed in conjunction with EAE, these include ultrasound, microwave, high-pressure processing and supercritical fluid pre-treatments. The synergistic effect between EAE and biomass pre-treatment with these green technologies has been shown to enhance protein extraction yield in addition to the yield of other compounds, as previously summarised by Nadar et al. (2018).

The application of ultrasound pre-treatment prior to EAE increased the protein yield from 61.60 to 69.80% (w/w) during Alcalase® 2.4 L assisted protein extraction from BSG (Yu et al. 2019). It was reported that pre-treatment with ultrasound reduced the enzyme dose and incubation time by 73 and 56%, respectively, while the protein yield was increased from 61.60 to 69.80% (w/w) when compared to extraction without ultrasound pre-treatment. This technique was also applied to aid protein extraction from sesame bran using Alcalase® (Görgüç et al. 2019a, b). The optimum conditions were reported to be: 836 W ultrasound power, an E:S of 1.488 AU/100 g and incubation for 98 min at 43 °C which resulted in a protein yield of 87.90% (w/w). This was compared to enzymatic extraction and ultrasound treatment alone which resulted in protein yields ranging between 41.00–79.00% (w/w). On the other hand, the combination of ultrasound-assisted enzymatic extraction (using AMG® 300 L) of the dried brown algal biomass, *Ecklonia cava*, yielded a significantly lower protein yield (15.08 mg/g) than that of the enzymatic (18.2 mg/g) or ultrasound (25.09 mg/g) treatment alone (Park and Lee 2021). This may be due to the effect of the heat generated during the ultrasound treatment which can contribute to denaturation of the enzyme and extracted proteins.

Microwave-assisted enzyme extraction was employed to aid protein extraction from sesame bran using Alcalase® 2.4 L, resulting in an increased protein yield from 62.30 to 91.70% (w/w) compared to microwave treatment alone (Görgüç et al. 2019b). An integrated microwave pre-treatment with an immobilised enzyme (Flavourzyme® 500MG on magnetic nanoparticles) process was employed to aid in the bio-refining of fresh pomegranate seeds. The results showed that this technique gave a significantly higher protein yield, i.e., 94.64% (w/w), when compared to the immobilised enzyme without microwave pre-treatment (65.48% (w/w)) (Talekar et al. 2020). In addition, it was demonstrated that microwave pre-treatment prior to enzymatic processing could reduce the enzyme dose required and shorten the reaction time, which can lead to a reduction in overall energy utilisation and process cost. Furthermore, the utilisation of the immobilised enzyme not only facilitated the reusability of enzyme but also maintained the stability of the enzyme activity over a broader pH range (pH 6.0–8.0) and at high temperatures

(up to 55 °C), while the use of free enzyme only yielded high recovery at specific conditions, i.e., pH 7.0, 45 °C (Talekar et al. 2020). This was in agreement with a related study which reported a process where co-immobilised Alcalase® and cellulase enhanced the extraction of oil from *Camellia sinensis* (Peng et al. 2019).

6.3.2.3 pH

There appears to be limited information regarding the effect of pH on the EAE of plant proteins. The optimal pH range of an enzyme preparation depends on the enzyme used, i.e., whether carbohydrases or proteases. Carbohydrases generally have an optimal pH's in the mildly acidic pH region, whereas the optimal pH of most commercially available food-grade protease preparations used during EAE is in the neutral to mildly alkaline pH range (Kumar et al. 2021).

Nonetheless, reaction pH is a key factor influencing enzyme-assisted protein extraction as it directly affects the activity of the enzyme(s). For instance, during Viscozyme® and Alcalase® EAE of protein from sesame bran, the higher the pH value the greater the protein yield observed (Görgüç et al. 2019a, b). Enzyme activity is highly dependent on the conformation of its active and allosteric sites, which in turn is strongly influenced by pH. Moreover, changing the pH from the optimal range can alter the conformation and the charge distribution of both the enzyme and the substrate. This may lead to a reduction in enzyme activity and binding affinity between enzyme and substrate (Singh et al. 2019). In addition, reaction pH was reported to have a major contribution to the extraction yield of specific proteins, e.g., during the extraction of R-phycoerythrin (R-PE) which is a major component of the light-harvesting pigment protein complexes, known as phycobiliproteins, found in red algae. The highest protein (0.32 mg/g dw) and R-PE (0.27 mg/g dw) yields were obtained at pH 7.1 compared to a lower pH extraction, i.e., at pH 6.5 (Nguyen et al. 2017). Furthermore, reaction pH not only affects protein yield but can also influence the functional properties of the recovered proteins (Rommi et al. 2015). Despite this, Benhammouche et al. (2021) demonstrated that pH (within the range pH 3.3–5.5) had no significant ($p > 0.05$) impact on the protein content extracted from defatted *M. oleifera* leaves when using Viscozyme® L.

6.3.2.4 Temperature

Temperature can affect the extraction process by "softening" plant cell tissue and by increasing solubility, which can enhance the recovery of protein and other components. However, temperature may also negatively influence protein stability due to the possibility for thermal degradation/denaturation (Görgüç et al. 2019a, b). This can, in turn, impact the technofunctional and bioactive properties of the extracted proteins/peptides. Nevertheless, the hydrolysis rate increases with increasing temperature (within the optimum temperature range) due to unfolding/loosening of substrate molecules resulting in more susceptibility to the enzyme(s) activities. This is particularly the case when proteases are being used (Singh et al. 2019).

In general, it is recommended to operate within the optimal temperature range for the enzyme/enzyme preparation used. Nonetheless, a lower or slightly higher operating temperature may be employed, however, this would require optimisation

in conjunction with other parameters on an enzyme-dependent basis. For instance, at temperatures above 45 °C, a significant reduction in protein extraction yield was observed in defatted soy pulp using Viscozyme® L (de Figueiredo et al. 2018), despite the optimum temperature being 50 °C (Table 6.3). Similarly, a higher protein yield (35.0 mg protein/g) was obtained from defatted *M. oleifera* leaves using Viscozyme® L when the reaction was performed at 30 °C for 0.5 h compared to incubation at a higher temperature for a longer time (50 °C x 6 h: 21.0 mg protein/g) (Benhammouche et al. 2021). Whereas protein extraction from sugar beet leaves using Pectinex™ Ultra SP-L showed a linear correlation between protein yield and incubation temperature and time (Akyüz and Ersus 2021). The incubation temperature also had an effect on the yield obtained from Alcalase® assisted protein extraction from sesame bran (Görgüç et al. 2019a, b) and from sacha inchi (*Plukenetia volubilis*) kernel cake (Chirinos et al. 2017). However, when hydrolysis temperatures above 48 and 70 °C were employed, no significant increase in protein yield was observed in the case of protein extraction from the sesame bran and from the sacha inchi kernel cake, respectively (Chirinos et al. 2017, Görgüç et al. 2019a, b).

6.3.2.5 Duration of Hydrolysis

Hydrolysis duration/incubation time is one of the most studied parameters during EAE of proteins. Initially, the rate of hydrolysis increases with increasing incubation time and subsequently decreases and then plateaus out, where no more products are generated (Singh et al. 2019). This may be due to depletion of the starting substrate, deactivation of the enzyme during long periods of incubation and the presence of higher levels of hydrolysis products which may act as enzyme inhibitors (Castro-Jácome et al. 2020). Furthermore, a long incubation period at high temperature can promote microbial growth. This may result in quality concerns during manufacturing. Whereas a shorter hydrolysis duration can be achieved by using a higher enzyme concentration or biomass pre-treatment in conjunction with physical approaches.

The following sections deal with EAE from a range of different plant sources.

6.3.3 EAE of Protein from Oilseeds and Nuts

Oilseeds are any seeds grown primarily for the production of edible oils. Soybean and rapeseed, along with sunflower, sesame, palm and cottonseed are the major oilseeds. The meal obtained from the oilseeds following the de-oiling process is a rich source of protein, however, the de-oiling process may have an adverse impact on the protein functionality. Therefore, a potential route to increase economic value for the oilseed industry relies on the ability to enhance the efficiency of the protein extraction process from the de-oiled seeds while retaining protein functionality and digestibility (Miquel et al. 2011).

Currently, the most widely used methodology to obtain protein fractions from the oilseed co-products (e.g., cakes and meals) involves alkaline solubilisation

(performed at pH 9–10 (Sari et al. 2015)), removal of the insoluble materials using centrifugation and isoelectric precipitation of the proteins followed by their separation using centrifugation. Pre-treatments prior to protein extraction such as the application of EAE may enhance protein extraction yield.

EAE of proteins is an eco-friendly, alternative process based on the simultaneous extraction of oil and proteins from oilseeds. A detailed description of the application of EAE for the separation of proteins and other components from oilseeds has been outlined previously (Liu et al. 2016). Briefly, this technique involves oilseed grinding, the enzyme treatment step with the addition of specific enzyme(s) followed by separation of the free oil, cream, solid and aqueous phases. The latter contains the proteins at the highest concentration. Subsequently, the aqueous phase is concentrated and dried as the protein fraction. These EAE methods have been established based on the specific modes of action by different enzyme activities which allow:

a) disruption of the integrity of cell walls: The major part of the oilseed cell wall is composed of proteins and carbohydrates (especially pectins) (Arrutia et al. 2020). Thus, the use of cell wall degrading enzymes, e.g., pectinases, is a promising strategy to facilitate the release of intercellular compounds. The role of most carbohydrases such as cellulases and pectinases in EAE processes is to disrupt the structure of cotyledon cell walls without mediating any adverse impacts on the protein structure.

b) degradation of the protein components to smaller fragments via *enzymatic hydrolysis:* This strategy involves hydrolysis of the proteins in cell membranes and inside the cytoplasm using proteolytic activities which catalyses the hydrolysis of peptide bonds in polypeptides. In addition to increasing the yield of protein extracted from the oilseeds, proteases may enhance protein solubility in the aqueous phase (Pojić et al. 2018). The use of proteases is generally more beneficial for low oil content materials (or de-oiled seeds) having a high protein content (Souza et al. 2019). For instance, protease preparations containing a mix of proteolytic activities, e.g., Protex 40XL®, Protex P®, Protex 5 L®, Protex 50FP® and Protex 26 L®, were successfully used for the extraction of proteins from rapeseed and soybean meals (Zhang et al. 2007).

c) degradation of antinutritional factors (ANFs): According to the literature, most of the oilseeds contain ANFs which may limit their applications for human and animal consumption by reducing protein digestibility. Furthermore, ANFs can have adverse effects on the technofunctional properties of the oilseed proteins. For instance, rapeseed contains phytic acid (1–3% (w/w)) which chelates minerals such as calcium, magnesium, zinc and iron, thus making them unavailable for absorption and metabolism (Tie et al. 2020). In addition, the presence of phytate in rapeseed may lead to the formation of insoluble phytate–mineral–protein complexes, which ultimately decreases the availability of some amino acids and reduces the exposure of the protein bonds to proteolytic degradation during digestion, consequently inhibiting the activity of some metabolic enzymes (e.g. trypsin, tyrosinase and pepsin) and thereby reducing protein digestibility. Glucosinolate is also present at high levels in rapeseed. This compound reduces protein digestibility. While the exact

mechanism is still unknown, it has been suggested that this adverse effect may be related to its influence on the ruminal microbiota (Gao et al. 2021). Another ANF in rapeseed is erucic acid which can be toxic for animals and humans at high consumption levels. Moreover, the presence of high contents of phenolic compounds (1–3% (w/w)) in rapeseed, which is ~10 times higher than in soybean, may result in reduced functionality such as solubility and protein digestibility via their interactions with proteinaceous components. Phenolic compounds interact with proteins either via non-covalent (such as ionic, hydrophobic and hydrogen bonding) or covalent (such as the formation of quinones following oxidation) interactions. These may reduce protein functionality, i.e., protein solubility and digestibility. Soybean also contains a diverse range of ANFs. The presence of trypsin inhibitors (e.g. Kunitz and Bowman–Birk inhibitors) reduces dietary nitrogen retention and therefore increases metabolic nitrogen excretion in humans and in animals (Arrutia et al. 2020). In addition, the presence of lectins in soybean, which bind to carbohydrates, is associated with reduced growth rates and increased mortality in animals. Furthermore, the presence of specific oligosaccharides such as stachyose, raffinose and verbascose (5.20% (w/w) in soybean compared to 2.20 and 1.60% (w/w) in sunflower and rapeseed, respectively) may reduce protein digestibility due to flatulence and intestinal hypertrophy in humans and in animals (Broudiscou et al. 2020). The presence of phytic acid in soybean, as outlined earlier, may also reduce the availability of minerals for absorption (Arrutia et al. 2020). Among the oilseeds, sunflower seeds have low amounts of ANFs while it has some phenolic compounds including non-esterified phenolic acids, isomers of caffeoylquinic, p-coumaroylquinic and dicaffeoylquinic acids, which are mostly located in the kernel (Karamać et al. 2012). The content of phenolic compounds in sunflower oil has been reported to be 2–4% (w/w) (Karamać et al. 2012). Accordingly, sunflower meals/cakes require less treatment to remove ANFs and phenolic compounds compared to, e.g., rapeseed and soybean meals/cakes during the extraction of their proteins. Consequently, due to the presence of high levels of ANFs and phenolic compounds in oilseeds, a specific strategy is required to obtain high protein yields and to increase the nutritional and technofunctional value of the extracted oilseed proteins. The impact of EAE on the allergenicity of plant proteins has been studied (Yu et al. 2011; Latif et al. 2013). While no impact of using Protex 6 L (50 °C, 2 h) during protein extraction from peanut has been observed on the levels of the main allergens, Ara h1, h3 and h6–8 (Latif et al. 2013), the use of α-chymotrypsin and trypsin (37 °C, 1–3 h) has been shown to reduce Ara h1 and Ara h2 levels in peanut kernels by 100 and 98%, respectively (Yu et al. 2011). Therefore, the use of enzymes with the capability of degrading ANFs and allergic compounds during protein extraction exemplify the benefits of employing EAE processes.

The differences in the composition of oilseeds determines the choice of enzymes used during EAE for each oilseed. Up to now, a large range of carbohydrase and protease enzymes have been utilised on their own during the EAE of proteins from oilseeds, as outlined in Table 6.4. However, some studies suggested that the use of a combination of enzymes with various activities is a more efficient approach to enhance the extraction of proteins from oilseeds (Arrutia et al. 2020).

Soybean is one of the most consumed oilseeds. The protein content in soybean meal is ~45% (w/w) (Ibáñez et al. 2020) and its nutritional quality is comparable with that of animal proteins. The protein digestibility corrected amino acid score (PDCAAS) of soybean proteins has been reported to be 1.0 (Khalesi and FitzGerald 2021). This makes soybean meal a low-cost alternative plant protein source to animal proteins. EAE has been reported as a promising technique for the extraction of high-quality proteins with good biological activity, nutritional quality and technofunctionality from soybean and soybean co-products. The utilisation of serine endo- and exo-proteases for the extraction of proteins from soybean has been shown to enhance protein yield by 10% compared to the extraction process without incubation of the samples with these enzymes (Sari et al. 2013). The use of 2.5% (w/w) Protex 6 L for the extraction of protein from flaked and extruded soybeans resulted in > 90% (w/w) protein recovery both at laboratory and pilot scale (De Moura et al. 2009). Zhang et al. (2019) showed that the use of Alcalase® 2.4 L and Protex 6 L in the extraction of proteins from soybean resulted in a higher extent of protein hydrolysis compared to using Flavourzyme and Protex 7 L. Another study reported that incubation of soy meal with a combination of enzymes including β-glucosidase, phytase and acid protease led to protein extraction yields of up to 75% (w/w) (Wei et al. 2018). In addition, Viscozyme® L has been used to extract protein (with a yield up to 83.3% (w/w)) from a soybean paste suspension (20% (w/v), pH 5.5) at an E:S 6% (v/w) (Penha et al. 2020). While it was also reported that the use of Viscozyme® L at a range of enzyme activities (i.e., 15–45 FBG/10 g solids) did not enhance protein yield (Rosset et al. 2014).

The use of a combination of cellulase, xylanase and pectinase followed by alkaline extraction of proteins from soy grits not only enhanced the protein yield by 13% (w/w) in comparison to alkaline extraction alone, but also resulted in improved protein functionality in terms of solubility, emulsification and foaming properties (Perović et al. 2020). Fischer et al. (2001) reported on the use of a combination of Alcalase (2.5% (v/w)) and Flavourzyme (5% (v/w)) at pH 7.6 to help extract soybean meal protein. The good emulsification properties of soybean protein extracted with the aid of proteolytic enzymes has been linked with greater exposure of hydrophobic amino acids, its enhanced surface hydrophobicity and interfacial adsorption due to protein unfolding. This was associated with the formation of small soluble aggregates following enzymatic modification (Lu et al. 2016). The proteins obtained following EAE were reported to have a lower level of trypsin inhibitors and phytic acid, and a higher functionality compared to conventionally manufactured soy protein concentrate (Zhang et al. 2019).

Rapeseed contains between 17–25% (w/w) protein depending on the variety and seasonal factors. The cell wall of rapeseed, which surrounds the proteins, is composed of a highly interconnected network created from different polysaccharides such as xyloglucan, xylan and pectin which are bound to each other and to cellulose. EAE of rapeseed proteins using multiple enzyme activities including Protex 7 L, Multifect Pectinase FE, Multifect CX 13 L and Natuzyme has been reported to improve protein extraction yield (Latif et al. 2008). It has been shown that between 15–30% (w/w) of rapeseed proteins may be extracted using the conventional

pH-shift method, whereas ~50–80% (w/w) of rapeseed proteins could be extracted using the EAE approach (Nadar et al. 2018). Kvist et al. (2005) also reported a protein yield of > 80% (w/w) from a wet-milled rapeseed enzyme treated sample using a combination of pectinase, β-glucanase and hemicellulase. Microstructural investigations demonstrated the presence of high amounts of pectin surrounding the proteins inside the embryo cells in rapeseed (Rommi et al. 2014). Accordingly, the use of pectinolytic enzymes (Pectinex) to hydrolyse the pectic polysaccharides and glucans caused disintegration of embryonic cell walls leading to a 1.7-fold enhanced protein release compared to the conventional extraction methods (Rommi et al. 2014). In addition, the combination of pectinolytic (Pectinex Ultra SP-L), xylanolytic (Depol 740 L) and cellulolytic (Celluclast® 1.5 L) enzymes has been reported to enhance the extraction of proteins from rapeseed (Rommi et al. 2014). Niu et al. (2012) reported that incubation of a dehulled, cold-pressed rapeseed press cake with a 1% (v/w) Viscozyme-Alcalase combination for 80 min resulted in a protein extraction yield of 82% (w/w). Furthermore, the use of a combination of Alcalase, cellulase, β-glucanase and pectinase for the extraction of proteins from rapeseed resulted in a protein yield of ~80–83% (w/w) (Zhang et al. 2007). It is reported that a cocktail of cellulase and protease activities is currently used at commercial scale for the extraction of proteins from rapeseed (Tang 2010). Moreover, a recent study showed that the use of proteases such as Protex 5 L, Protex P and Protex 40XL which work optimally at alkaline pH resulted in a higher protein recovery compared to proteases having optimum pHs under mildly acidic conditions, e.g., Protex 26 L and Protex 50FP (Sari et al. 2013). The application of phytase during EAE has also been reported to improve the extraction of proteins from rapeseed (Rodrigues et al. 2017). This has been linked with the degradation of phytate which is capable of interacting with the proteins, thus obstructing their enzymatic degradation and reducing the bioavailability of their amino acids.

Sunflower meal contains ~25% (w/w) protein and is mainly used for ruminant feed. Yust et al. (2003) improved the alkaline protein extraction process from sunflower meal by incubation of the meal with Alcalase, which resulted in a higher protein yield (87.40 vs 57.50%) and better protein solubility (4.5 times) compared to conventional extraction methods.

Sesame is an oilseed crop with 22–25% (w/w) protein consisting of globulins, albumins, prolamin and glutelin. Sesame protein has a high methionine content (3.2% (w/w)) which makes it an attractive protein source for use in several food applications. EAE using 2% (w/w) Protex 7 L, Alcalase® 2.4 L, Natuzyme, Kemzyme and Viscozyme® L was performed to extract sesame seed protein with the use of Protex 7 L at optimum conditions (E:S 2.0% (w/w), 45 °C, pH 7, 2 h) leading to the highest protein yield at 87.10% (w/w) (Latif and Anwar 2011).

Flaxseed meal contains 35–40% (w/w) protein. Defatted flaxseed meal subjected to alkaline extraction and isoelectric precipitation (at pH 5.0) followed by incubation with cellulase at optimum conditions (E:S 2.0% (w/w), 37 °C, pH 5.0, 4 h) led to a protein recovery of 65% (w/w) (Tirgar et al. 2017). The recovered proteins had higher solubility (95% at pH 7.0 and 80% at pH 2.0) compared to the conventionally extracted protein (90% at pH 7.0 and 70% at pH 2.0) and this was linked to protein

structure reorganisation following enzymatic treatment which resulted in a reduced hydrophobic patch on the protein surface (Tavel et al. 2008).

Palm kernel contains 14–20% (w/w) protein. The utilisation of trypsin (E:S 1.40% (w/w), 37 °C, pH 9.5, 6 h) during the extraction of proteins from palm kernel significantly enhanced protein yield (up to 72% (w/w)) compared to the conventional alkaline extraction method (10% (w/w)). The resultant protein extract following EAE had a higher in vitro digestibility, essential amino acid (EAA) content and solubility but lower emulsification and foaming properties compared to soybean protein isolate (Chee et al. 2012).

Nuts such as almond, walnut, hazelnut, pistachio, peanut and cashew have ~10–26% (w/w) protein (Brufau et al. 2006). Almost all nut-derived proteins have low levels of threonine and isoleucine, but contain high levels of tryptophan and leucine. Walnuts and peanuts have a higher protein content compared with other popular nuts. Each nut contains a large variety of proteins, for instance, 188 different proteins have been detected in almond seeds with amadine (from the family of 11S globulin) as the main fraction, while the major protein in walnuts is glutelin (Qamar et al. 2020). The use of Alcalase-assisted extraction of walnut proteins resulted in a protein yield of 67% (w/w) compared to 39% (w/w) yield using the conventional alkaline extraction method (Hu et al. 2017). The structural modification obtained as a result of Alcalase-assisted protein extraction resulted in higher solubility. In addition, the use of FoodPro® Alkaline Protease, a commercial bacterial alkaline endoprotease from *B. licheniformis*, at an E:S of 0.85% (v/w), for the extraction of proteins from almond cake (having 37% (w/w) protein) resulted in a protein yield of 75% (w/w), which was higher than the yield obtained with the alkaline extraction process (70% (w/w)) (Souza et al. 2019). The extraction of proteins from Tiger nut with the use of a mixture of Alcalase, α-amylase, Viscozyme® L and Celluclast® 1.5 L gave the highest yield compared to using the individual enzymes (Ezeh et al. 2016). The extraction of pine seed proteins with the aid of Alcalase (E:S 1.9% (v/w), 55 °C, pH 8.8, 2.2 h) resulted in 88% (w/w) protein extraction (Wang et al. 2011). Chirinos et al. (2017) showed that the Alcalase-assisted extraction at the optimum conditions (E:S 5.60% (w/w), 50 °C, pH 9.0, 40 min) resulted in 45% (w/w) protein recovery from sacha inchi kernel which was higher than that obtained with the alkaline extraction process (29.7% (w/w)).

Among the nuts studied, the use of EAE with peanuts appears to be the most extensively investigated. Peanuts contain ~26% (w/w) protein. De-oiled peanut flour is a protein-rich, inexpensive product which has rarely been used for human consumption until recently. However, it contains 47–55% (w/w) protein with a high EAA content which may be useful for different food applications (Ma et al. 2010). Among the different proteolytic enzymes (Alcalase, AS1398, Nutrase, Protizyme and Protamex) tested for the EAE of protein from peanut, Alcalase and Protamex gave the maximum (73% (w/w)) and the minimum (49% (w/w)) protein yields, respectively. Incubation of peanut meal at the optimum conditions using Alcalase (E:S 1.5% (w/w), 60 °C, pH 9.5, 5 h) led to a protein extraction yield of 88.21% (w/w). This protein extract possessed high 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) scavenging and angiotensin-I-converting enzyme (ACE) inhibitory activity

(Jiang et al. 2010). During the extraction of proteins from roasted (190 °C for 20 min) peanut seeds, the use of Alcalase® 2.4 L (E:S 2.00% (v/w), 55 °C, pH 9.0, 3 h) yielded protein hydrolysates with a protein equivalent content of 80.10% (w/w) (Zhang et al. 2011). Protex 6 L-assisted aqueous extraction (E:S 0.50% (w/w), 50 °C, pH 8.0, 2 h) of peanut kernel gave a protein extract with higher quantities of EAAs, reduced trypsin inhibitor activity and lower phytate content compared to non-enzyme extraction processes (Latif et al. 2013). The impact of EAE using various carbohydrases including Viscozyme® L, cellulase, hemicellulase and pectinase on peanut protein yield and protein functionality has been investigated. Among the enzymes tested, incubation of peanut meal with Viscozyme® L (E:S 1.35% (w/w), 52 °C, pH 7.2, 1.5 h) resulted in the highest protein recovery (79% (w/w)) with the extract having improved solubility, foaming and emulsification properties compared to commercial peanut proteins (Liu et al. 2020). Furthermore, it has been shown that the application of EAE using Protex 6 L (E:S 0.5% (w/w), 50 °C, pH 8.0, 2 h) reduced the trypsin inhibitory activity of the peanut protein extract (Latif et al. 2013). The authors, however, identified allergen proteins including Ara h1 (DLAFPGSGEQVEKL), Ara h3 (RSVNELDLPIL), Ara h6 (KRELMNLPQ), Ara h7 (ELRNLPQ), Ara h 8 (KPDEEELK) and Ara h8 isoform (KATVVDGDELTPK) in the peanut protein extracted via EAE.

6.3.4 EAE of Protein from Pulses

According to the Food and Agricultural Organisation (FAO 2016), pulses are the edible seeds of leguminous plants that are harvested solely for the dry seed or grains, including: dried beans (genera *Phaseolus* and *Vigna*) such as pinto, black and white beans; chickpeas (genera *Cicer*); lentils (genera *Lens*) and peas (genera *Pisum*). Pulses constitute an important source of dietary protein and their protein content can range between 21 and 30% ((w/w) dw), which varies with the variety, germination, growth environment (Singh 2017) and cooking (de Almeida Costa et al. 2006). Based on Osborne's fractionation, pulse proteins are classified as albumins (water-soluble), globulins (salt soluble), prolamins (alcohol soluble) or glutelins (soluble in dilute acid or alkali detergents) (Bessada et al. 2019). Globulins (mainly 7S and 11S) are the most abundant proteins in pulses, constituting up to 91% (w/w) of the total protein (Mundi and Aluko 2012). Albumins are the second most abundant proteins (8–30% (w/w) of the total protein) and this fraction includes enzymes, protease inhibitors, amylase inhibitors and lectins (Bessada et al. 2019).

The structure of different legume seeds is reported to be very similar; the cotyledon architecture is composed of starch granules embedded in a matrix of protein bodies which are surrounded by a fibre-rich cell wall (Pelgrom et al. 2015). Conventionally, different techniques have been employed to extract proteins from pulses, including: alkaline extraction/isoelectric precipitation, air classification, acid extraction, water extraction, salt extraction and ultrafiltration (Boye et al. 2010). The recovery yield of total protein, the protein content and the chemical composition of the resultant product depends on the method and conditions used. For example,

protein extracted from fava bean and pea flours using alkaline extraction can reach protein concentrations between 70 and 93% ((w/w), N x 6.25) (Paredes-López et al. 1991, Fernández-Quintela et al. 1997, Felix et al. 2019, Anusha et al. 2021), with recovery yields of 58 and 65% (w/w) protein obtained for fava bean and pea flours, respectively (Gueguen 1983). The use of drying processes such as micronisation and air classification has generated protein fractions with protein contents of 49, 57, 56 and 47% ((w/w) N x 6.25) for red lentil, yellow lentil, green pea and chickpea, respectively (De Angelis et al. 2021) and 56, 53, 45 and 59% ((w/w), N x 6.25) for pea, bean, chickpea and lentil, respectively (Pelgrom et al. 2015). However, information on extraction yield is more limited. The application of EAE to pulses can result in hydrolysis of the cell wall and the release of proteins. The EAE of pulse proteins employs three main strategies, i.e., extraction with carbohydrases, proteases or a combination of both carbohydrases and proteases.

However, the current literature in relation to the use of EAE as a complementary/alternative method for pulse protein extraction with carbohydrases appears to be limited (Table 6.4), likely due to the high yield obtained with the traditional wet and air classification methods. Bildstein et al. (2008) employed different cell wall degrading enzymes such as α-amylases, glucoamylases, pectinases, cellulases and xylanases to increase protein recovery in lentil and white beans in comparison with water extraction alone. The extraction of milled lentil and white bean with glucoamylase (Distizym® AG), E:S 0.10% (v/w), pH 7, 50 °C for 2 h with constant stirring provided the highest protein recovery, i.e., 36 and 72% (w/w) for lentil and white bean, respectively. This was 19% higher than the yield obtained using aqueous extraction alone (Bildstein et al. 2008). Carbohydrases, i.e., α-amylase and amyloglucosidase, have also been used to hydrolyse milled Borlotti beans to increase the bioaccessibility of its polyphenols (Perez-Hernandez et al. 2020). While fava bean flour has been hydrolysed with α-amylase to remove the starch and improve its emulsifying properties (Jiang et al. 2020). de la Rosa-Millán et al. (2019) analysed the impact of jet-cooking followed α-amylase or isoamylase aided protein extraction from chickpea flours at pilot scale. The results showed no differences in protein composition with an increase in in vitro digestibility when carbohydrases were employed for both raw and jet cooked flour.

The literature in relation to the use of proteases is more abundant. However, the focus of most of these studies is to improve the functional properties and/or to characterise the bioactivities of the resultant protein hydrolysates and, therefore, the information in relation to the protein yield and content is limited. For example, the potential bioactive peptides released following protease hydrolysis of chickpeas have been reviewed (Real Hernandez and Gonzalez de Mejia 2019), however, no information in relation to protein yield was included. Table 6.4 summarises the reports which used EAE with proteases during protein extraction from pulses. In most cases, the starting material is the grain or its flour which are extracted using water/alkaline extraction followed by protein concentration/purification by isoelectric precipitation resulting in a protein concentrate or isolate. Sometimes the flour or protein concentrate is cooked at 80 °C to inactivate endogenous enzymes (Ghribi et al. 2015). Subsequently, the proteins are digested with proteases, with Alcalase

being the enzyme most used in the literature reports. Examples of protein hydrolysates obtained with Alcalase have been described for lentils (Xu et al. 2021), chickpeas (Clemente et al. 1999; Ghribi et al. 2015; Felix et al. 2020; Quintero-Soto et al. 2021; Xu et al. 2021), beans (Samaei et al. 2020; Castañeda-Pérez et al. 2021), pigeon peas (Xu et al. 2021) and peas (Li and Aluko 2010). The hydrolysis of chickpea protein concentrate (Felix et al. 2020) or faba bean protein concentrate (Felix et al. 2019) with Alcalase has been reported for, e.g., producing hydrolysates with improved emulsifying, antioxidant and ACE and dipeptidyl peptidase-IV (DPP-IV) inhibitory activities. Moreover, different proteases have been employed to obtain protein hydrolysates from chickpea and pea by-products with degree of hydrolysis (DH) values ranging from 12.40 to 42.90%, with protein extraction yield values ranging from 12 to 58% (w/w) and with protein content values ranging from 35.4 to 66.0% (w/w) (Prandi et al. 2021).

Finally, simultaneous digestion with carbohydrases and proteases has been employed with chickpea flour to obtain beverages having high levels of soluble protein and high TS (Silvestre-de-León et al. 2020, 2021) (Table 6.4). The instant beverage-based powders obtained after simultaneous digestion of chickpea flour with Alcalase® 2.4 L and Thermozyme® L340 increased the soluble protein content by 43% in comparison to the supernatant obtained without hydrolysis (20.77 vs 29.72% (w/w) on a dry–matter basis in relation with the total protein in the dry sample). When extrusion was carried out before the simultaneous digestion, the increase in soluble protein was even higher with values ranging from 56.29 to 70.03% (w/w). The chickpea flours obtained displayed improved in vitro digestibility and technofunctional properties (Silvestre-de-León et al. 2020).

6.3.5 EAE of Protein from Cereals and Grains

Cereals and grains (the edible part of the cereal), e.g., wheat, barley, rice, oat, rye or maize, are one of the most basic ingredients in the human diet and in animal feed with estimated worldwide production of 2769.4 million tonnes in 2020 (FAO 2022). Cereals and grains are a good source of proteins with contents ranging from 5.8 to 15% (w/w) in the case of crude grains (Shewry 2007) while their by-products (e.g., spent grains) can contain protein contents between 30 and 85% (w/w). Despite their high protein content, in general, cereals and grain proteins have a relatively low nutritional quality due to their lack/low levels of specific EAAs such as tryptophan, methionine and lysine (Vaclavik and Christian 2014). However, due to their intrinsic physicochemical properties, cereals are mainly used as the main ingredients to improve the technofunctional properties of final products such as bread and pasta.

Grains are composed of the endosperm, germ and bran. In the case of whole grains, the three components remain, while in the refined grains only the endosperm is maintained. There are four main proteins in cereal grains, i.e., albumins and globulins (stored in the germ), gluten-derived proteins (gliadin and glutenins) and prolamins (stored in the endosperm) (Tapia-Hernández et al. 2019). The proteins in cereal grains are mainly classified based on their solubility. For instance, albumins

are water soluble while glutenins are soluble in alkali, globulins are soluble in salt solutions and prolamins are soluble in alcohol solutions. Since the solubility of these proteins in aqueous solutions is relatively low, new techniques such as EAE of protein have been investigated, and these are currently being used in order to improve their solubilisation and extraction in a sustainable manner (Bozkurt et al. 2021).

The structure of cereal grains is complex. The proteins are usually located in the endosperm of the grain and they are covalently cross-linked within the carbohydrate network hindering their accessibility. pH-shift techniques, i.e., alkaline solubilisation and isoelectric precipitation, are effective in cleaving these bonds with polysaccharides leading to the release of protein. However, these extraction approaches are not ideal due to the incorporation of high salt content, the formation of lysinoalanine at pH values of 10 or higher (reducing the nutritive value) and, in some instances, the protein yield can be low (Guan and Yao 2008; Deleu et al. 2019). This has been attributed to the high amount of fibre which increases the viscosity of the solution and, therefore, the pH can be heterogeneously distributed thereby reducing protein precipitation at their isoelectric points (Jodayree et al. 2012). In this instance, the use of carbohydrases has been proposed as an alternative in order to break the plant cell wall structure and to release the proteins without the use of strong alkali. Some examples of EAE of protein from cereals and grains are summarised in Table 6.4. Jodayree et al. (2012) used four carbohydrases under different conditions and improved the protein content of oat bran isolates, with the highest increase being from 54 to 82% (w/w) when using amyloglucosidase. Similarly, a pre-treatment of oat bran with Viscozyme® L prior to alkaline extraction yielded protein content values of 56.20% (w/w) while when using alkaline extraction alone the protein content achieved was 14.80% (w/w) (Guan and Yao 2008). Houde et al. (2018) improved the extraction of barley proteins and increased protein yield by 25% by using a combination of 3 carbohydrases: α-amylase (E:S 10,000 U/g, 65 °C, pH 6.5, 1 h), amyloglucosidase (E:S 660 U/g, 40 °C, pH 6.5, 16 h) and β-1,3,4-glucanase (E:S 8 U/g, 37 °C, pH 5.0, 1 h).

The application of proteases in cereals improves protein solubility and, therefore, enhances their extraction. Furthermore, enzymatic hydrolysis with proteases leads to an improvement in protein technofunctional and bioactive properties and in some cases a decrease in the allergenicity of several proteins such as the glutenins (Pourmohammadi and Abedi 2021). The use of four different enzymes (Flavourzyme, Alcalase, Savinase and Subtilisin) to achieve two DHs was investigated with the aim of improving the solubility and, thereby, the extraction efficiency and the functionality of wheat glutenins. The authors reported that all the hydrolysates with low DH had improved technofunctional properties (Bozkurt et al. 2021). A direct protease extraction approach was followed during the extraction and solubilisation of protein from rice dreg (a by-product of rice syrup processing). The authors reported that the Alcalase and trypsin hydrolysates resulted in the highest protein recoveries of 58 and 57% (w/w), respectively (Li et al. 2012).

As mentioned elsewhere, the use of both carbohydrases and proteases has been shown to positively affect the extraction of proteins. In some occasions, the use of a

direct enzymatic approach avoids the use of strong alkali solutions while resulting in similar protein contents. For instance, Connolly et al. (2019) reported the use of a combination of carbohydrases and proteases for the extraction of protein from BSG. Similar protein contents were observed in the resultant hydrolysates generated from a carbohydrase in conjunction with protease aided BSG protein extract (direct enzymatic approach) and a BSG protein isolate obtained using the pH-shift approach (44.65 ± 0.27 and 46.16 ± 4.20% protein, respectively). The protein yield with the best combination of enzymes (Shearzyme + UltraFlo and Alcalase + Flavourzyme) was 63.09% (w/w) compared to 58.90% (w/w) obtained from the pH-shift approach. A combination of carbohydrases and proteases was also investigated in the release of protein from rice dreg. The application of Viscozyme® L and Termamyl 120 along with hydrolysis using Alcalase gave the highest protein recovery at 71.83% (w/w) (Zhao et al. 2012).

Finally, the combination of a pH-shift and enzymatic (carbohydrase and/or proteinase) approach seems to be the most effective technique to extract protein and improve its characteristics. A frequently used approach reported in the literature consists in the isolation of protein by pH-shift extraction along with a further incubation of the extract with carbohydrases and proteases to further extract and solubilise the protein. For instance, Aluko and Monu (2003) extracted protein from the pseudocereal quinoa using a pH-shift approach (yielding a protein concentrate) followed by hydrolysis with Alcalase (yielding a protein hydrolysate) and they observed greater solubility in the hydrolysate compared to the concentrate. However, the combined pH-shift and enzyme hydrolysis method involves additional centrifugation steps during processing which could render the process more expensive and potentially less sustainable.

6.3.6 EAE of Protein from Algae

Several macro- and microalgal species are rich sources of high-quality proteins (Becker 2007; Harnedy and FitzGerald 2011; Bleakley and Hayes 2017). However, access to algal proteins is hindered by the presence of the polysaccharide-rich cell wall, the location of proteins within macromolecular cell wall assemblies and the manner in which they are bound to polysaccharides within these assemblies (e.g. cross-linked via disulphide bonds). Furthermore, the extraction of algal proteins is impeded by the high viscosity and the interactions with intracellular polysaccharides along with interactions with other non-protein components such as polyphenols within the algal cell.

Enzymatic pre-treatment with polysaccharidases, such as cellulases, hemicellulases, β-glucanases, xylanases and lysozyme and combinations thereof has been identified as a promising food-grade approach to break down algal cell walls and the intracellular polysaccharides to facilitate the extraction of algal proteins (Bleakley and Hayes 2017). However, careful selection of specific enzymes for each algal species is required as the constituent cell wall and intracellular polysaccharides differ significantly across algal species. Proteolytic enzymes,

alone or in combination with polysaccharidases have also been utilised for algal protein recovery.

In general, red macroalgae contain higher levels of protein compared to green and brown species with levels of 35 and 47% (w/w) reported for *P. palmata* (dulse) and *Porphyra tenera* (nori), respectively (Harnedy and FitzGerald 2011). As a result, the application of enzyme-assisted protein extraction has been studied largely with the red species. To date, polysaccharidases such as xylanases, cellulases, hemicellulase, β-glucanases and arabinases have been successfully used for the extraction of proteins and specific protein components from *P. palmata* (Fleurence et al. 2001; Joubert and Fleurence 2008; Harnedy and FitzGerald 2013; Maehre et al. 2014; Bjarnadóttir et al. 2018; Naseri et al. 2020). These polysaccharidases and combinations thereof were used for targeted hydrolysis of β-(1,3)/β-(1,4)-D-xylans and cellulose which are found in the cell wall of this species (Harnedy and FitzGerald 2013). Harnedy and FitzGerald (2013) and Maehre et al. (2014) both assessed the impact of using a combination of a cellulase and a xylanase for enzymatic pre-treatment of *P. palmata* cell walls prior to protein extraction using the standard alkaline extraction method. In both studies, an approximate 1.65-fold increase in the concentration of protein extracted was observed using cellulase/xylanase-assisted cell disruption compared to that obtained with osmotic shock induced cell disruption alone with Harnedy and FitzGerald (2013) reporting a protein recovery yield of approximately 67% (w/w). Similarly, Joubert and Fleurence (2008) reported an increase in the yield of aqueous soluble proteins extracted using cellulase/xylanase-assisted cell disruption (1–2 mg protein/g dw) compared to the control without added enzymes (0.40–0.75 mg protein/g dw). Furthermore, it was found that cellulase/xylanase-aided extraction improved the yield of R-PE which is an industrially relevant pigmented phycobiliprotein from 0.05–0.10 to 0.20–0.30 mg R-PE/g dw (Joubert and Fleurence 2008). Dumay et al. (2013) also reported that enzymatic pre-treatment with xylanase increased the yield of R-PE (3.28 g/kg dw) extracted compared to extraction without enzymatic pre-treatment (0.20 g/kg dw). Following optimisation using RSM, the quantity of R-PE extracted increased to 12.36 g/kg dw. Optimisation of the EAE process also increased the purity of R-PE by a factor of 2.9 (Dumay et al. 2013). Two further studies investigated the use of polysaccharidase and proteolytic enzyme preparations, used on their own and in combination, for EAE of proteins from *P. palmata*. In the first study, Bjarnadóttir et al. (2018) utilised a bacterial-derived thermostable recombinant xylanase and the commercial proteolytic preparation Umamizyme™, which contains both endo-proteinase and exo-peptidase activities, to aid protein extraction. Following enzymatic treatment, the mixture was separated into a liquid fraction and a solid residue using a 100 μm sieve. On a nitrogen basis (comparison to the nitrogen content of the raw material which was 4.10 ± 0.50% (w/w) dw), the extraction yield in the soluble fraction following pre-treatment with xylanase (24.40% (w/w)) was similar to that observed with the control where no enzyme was added (19.50% (w/w)) and protein yields of 62.20 and 59.91% (w/w) were observed, respectively, in the corresponding non-soluble fractions obtained after separation (Bjarnadóttir et al. 2018). However, when Umamizyme™ was used

alone and in combination with the xylanase, the yield in the liquid fraction increased to 63.40 and 66.60% (w/w), respectively, and the yield from the non-soluble fractions in both cases was approximately 21.00% (w/w) (Bjarnadóttir et al. 2018). In a second study, Naseri et al. (2020) assessed the effect of using enzyme preparations containing a xylanase (Shearzyme® 500 L), a cellulase (Celluclast® 1.5 L) and Viscozyme® L (a multi-complex polysaccharidases) and the proteolytic preparation Alcalase® 2.4 L on protein extraction efficiencies from *P. palmata*. In all cases, where polysaccharidases were used alone or in combination with Alcalase® 2.4 L, the proteins were subsequently extracted by alkaline solubilisation following enzymatic pre-treatment. After alkaline solubilisation the suspension was separated into a solid residue and liquid phase and the protein in the liquid phase was precipitated by isoelectric precipitation at pH 3.0. In terms of the quantity of protein extracted, the highest yields were observed when Alcalase® 2.4 L (0.20% (w/w)) was used in combination with either Celluclast® 1.5 L (90.00% (w/w)) or Shearzyme® 500 L (85.50% (w/w)). However, the concentration/purification of the extracted protein by isoelectric precipitation proved challenging as 70.00% (w/w) of the protein, present as peptides, remained in the liquid phase following isoelectric precipitation at pH 3.0. Mendez and Kwon (2021) assessed the effect of using the commercial cellulase preparations, Viscozyme® L and Celluclast® 1.5 L (2% (v/w)), in combination with aqueous-alkaline and sequential protein extraction approaches, to optimise crude protein recovery from the Pacific dulse (*D. mollis*). Following enzyme-assisted cell wall degradation, the highest protein yields (Viscozyme® L: 67.12% (w/w) and Celluclast® 1.5 L: 80.27% (w/w)) were recovered using a sequential water-, saline-, alkaline- and ethanol-soluble protein extraction approach at a temperature of 37 °C (Mendez and Kwon 2021). Furthermore, a high yield of protein was also obtained when the extraction was performed at 7 °C using the enzymes Viscozyme® L: 58.80% (w/w) and Celluclast® 1.5 L: 74.04% (w/w).

In addition to cellulose, the main polysaccharides found in the cell wall of the red macroalgae *Chondrus crispus* and *Gracilaria verrucosa* are carrageenan and agar, respectively. The combined application of carrageenase and cellulase on *C. crispus* and agarase and cellulase on *G. verrucosa* resulted in a ten-fold and three-fold increase in protein yield, respectively, compared to the yield where no enzyme was added (Fleurence et al. 1995). Like *P. palmata*, *Porphyra* sp. produces phycobiliproteins. These pigmented molecules spontaneously fluoresce in vitro and in vivo and have wide applications in the biotechnology (e.g., as probes) and food industry (as natural colourants). Furthermore, phycobiliproteins are reported to exhibit various biological activities (Harnedy and FitzGerald 2011). Using marine-derived bacterial cultures with induced agarase, amylase, cellulase, xylanase, carrageenase and mannanase activity, Huang et al. (2021) developed an effective EAE procedure for the extraction of the phycobiliproteins, phycoerythrin and phycocyanin from *Porphyra* sp.. Levels of 0.17 mg R-PE and 0.22 mg R-PC were determined per mL of the liquid fraction following centrifugation of the carbohydrase-treated sample, with purities of 0.33 and 0.23 reported for R-PE and R-PC, respectively.

Cellulase-assisted protein extraction (using the commercially available CellicCTec3® preparation which in addition to cellulase contains hemicellulase and β-glucosidase activity) from the red and brown seaweeds, *C. chamissoi* and *M. pyrifera,* led to significantly higher protein yields compared to the yields obtained using non-enzymatic processes (Vásquez et al. 2019). Following optimisation of the extraction process (at a constant temperature of 50 °C) using a central composite experimental design approach, protein yields of 74.60 and 36.10% (w/w) were obtained for *C. chamissoi* and *M. pyrifera*, respectively.

A comparative protein extraction study evaluated the use of six non-selective commercial enzymatic preparations, two protease (a neutral and a combination of neutral-alkaline proteases) and four carbohydrase (C1: cellulase, endo-1,4-β-xylanase, endo-1,3(4)-β-glucanase and feruloyl esterase; C2: endo-1,4-β-xylanase and endo-1,3(4)-β-glucanase; C3: a cellulase and C4: an exo-β-1,3 (4)-glucanase) preparations as a tool for improving protein extraction efficiency from *Ulva armoricana* (Hardouin et al. 2016). The extraction efficiency was determined by comparing the protein yields following enzyme-assisted extraction to those obtained following incubation of the macroalgal biomass with no enzyme under the same conditions. The results indicated that treatment with the neutral protease significantly increased the protein extraction yield (41.39% (w/w)) compared to the non-enzyme treated control (38.50% (w/w)), and the combination of neutral-alkaline proteases (26.64% (w/w)) and the carbohydrase-treated extracts (25.41–34.84% (w/w)).

Only a limited number of microalgal species, including *Spirulina* sp., *Chlorella* sp., *Dunaliella sp.*, *Aphanizomenon sp.* and *Nostoc* sp., are used for human consumption due to strict food safety regulations and market demand (Rizwan et al. 2018). However, some microalgal species, such as *Chlorella vulgaris* and *Spirulina maxima*, are a rich source of protein with levels of 58 and 71% (w/w, dw) reported for each, respectively (Barkia et al. 2019). EAE has also been used during the extraction of proteins from microalgal cells.

Spirulina sp. has a cell membrane similar to that of Gram-negative bacteria, in that they have two lipid layers (cellular and cytoplasmic), which are divided by a rigid murein layer made up of complex polymers of peptidoglycans and lipopolysaccharides. Both the cellular and cytoplasmic membranes contain proteins that are linked to lipids, in the former by non-covalent links and in the latter by covalently links. Verdasco-Martín et al. (2019) selected two proteolytic preparations (Alcalase® 2.4 L and Flavourzyme®) and two endo-/exo-glucanase preparations (Ultraflo® L and Vinoflow® Max A) to target the hydrolysis of specific molecules within the cell membrane and assessed the effect of these enzyme preparations in aiding the recovery of proteinaceous components from *Spirulina platensis.* Recovery yields were determined based on total amino acid profiles in the extracts, with Alcalase® 2.4 L the only enzyme shown to mediate an increase in yield compared to the non-enzyme treated control (Verdasco-Martín et al. 2019). However, the proteinaceous extract recovered contained mainly short peptides and free amino acids. In a separate study, a protein yield of approximately 82.00% (w/w) was obtained from *S. platensis* following enzymatic treatment with cellulase (Mahali and Sibi

2019). On comparison to other methods of protein extraction (alkali, thermal, microwave- and ultrasound-assisted extraction) EAE was reported to yield the second highest recovery, with ultrasound-assisted extraction yielding the highest, with a protein yield of 84.00% (w/w) and protein yields of 75.00, 64.00 and 79.00% (w/w) reported for alkali, thermal and microwave-assisted extraction, respectively (Mahali and Sibi 2019).

Duangsee et al. (2009) and Devi et al. (2020) both assessed the effect of using lysozyme for enzymatic pre-treatment of *Spirulina* sp. cells during the extraction of phycobiliproteins. Lysozyme, which mediates bacterial cell wall lysis through cleavage of peptidoglycan components, was selected to cleave the peptidoglycan layer in *Spirulina* sp. cell walls. Pre-treatment of *S. platenis* cells with lysozyme yielded a phycocyanin recovery similar to that obtained with ultrasound-assisted extraction and this was higher than that obtained with freeze-thaw assisted extraction (Duangsee et al. 2009). An extraction yield of 76.30 and 72.50% (w/w) was reported for lysozyme-assisted extraction of C-phycocyanin and allophycocyanin from *S. maxima*, respectively (Devi et al. 2020). However, this was lower than that obtained with ultrasound-assisted extraction, where extraction yields of 93.00 and 75.00% (w/w) were reported for C-phycocyanin and allophycocyanin, respectively.

In addition to *Chlorella* sp., lysozyme was reported to assist in the extraction of protein from other microalgal strains including *Chlamydomonas* sp., *Scenedesmus* sp. and a mixed culture (M.C. sp.) with protein yields from 79.00 to 97.00% (w/w) being reported (Al-Zuhair et al. 2017). Furthermore, cellulase was effective in aiding the extraction of protein from *Chlamydomonas* sp., *Chlorella* sp. and M.C. sp. (0.50–0.70 mg protein/mg dw) (Al-Zuhair et al. 2017). Cellulase from *Trichoderma reesei* ATCC 26921 was also shown to aid in the extraction of protein from *Chlamydomonas mexicana* when used in combination with ultrasound (Eldalatony et al. 2016). Combined sonication and hydrolysis treatment released 7.30% (27.1 ± 0.9 mg/g dw) soluble protein.

Bacterially-derived proteolytic enzymes were used to assist the extraction of protein from the green microalgae *Chlorella fusca* biomass and *C. fusca* meal (Sari et al. 2013, 2016). On comparison with the conventional alkaline-assisted extraction method, the protein yield with the EAE increased from 17.60 to 49.10% (w/w) and from 31.70 to 73.20% (w/w) for microalgae and microalgae meals, respectively (Sari et al. 2016). It was also reported that oil removal prior to the extraction process was beneficial for protein recovery (Sari et al. 2013).

The proteolytic enzyme preparation Alcalase® 2.4 L was used to assist in the extraction of proteins from *Nannochloropsis gaditana* (Safi et al. 2017). While a protein extraction yield of 35.00% (w/w) was obtained, flow cytometry analysis indicated that the extracted peptide components were more than likely derived from cell wall proteins as the cell wall remained intact. This would indicate that under the conditions assessed, Alcalase® 2.4 L was unable to completely weaken the integrity of the microalgal cell wall and release the majority of the intracellular proteins.

It must be noted that while proteolytic preparations when used alone or in combination with polysaccharidases result in an increased protein extraction yield from algae, the proteins extracted are in peptide format and not as intact proteins.

The downstream applications of the extracted algal proteins, whether in intact protein or shorter peptide format, in addition to the purity of the protein/peptide fraction (which cannot be precipitated by isoelectric precipitation) will govern whether proteolytic enzymes can be utilised for the extraction process. It must also be noted that if the intended downstream application requires proteins to be present in an intact format, polysaccharidase preparations which contain endogenous proteolytic activities, e.g., Ultraflo™ L, should be avoided (Harnedy and FitzGerald 2013). On the other hand, if the desired product is an algal protein hydrolysate, the application of proteolytic enzymes may be advantageous as both the enzymatic hydrolysis process and release of protein fragments are achieved in a single process. Furthermore, the purity of the peptide fraction released can be increased through the utilisation of membrane technologies such as ultrafiltration.

6.4 Conclusion and Future Perspective

EAE is one of the most promising green approaches used to aid plant protein extraction. This is due to it being eco-friendly (does not require harsh chemicals or very high temperature treatments), non-toxic (it can improve digestibility and decrease ANF compounds along with reducing allergenic potential) and is highly amenable to scale up. A range of commercially available food-grade carbohydrases and proteases are currently employed during EAE of plant protein-rich substrates. They are mainly in the form of a mixture of carbohydrases which have a range of specificities along with broad specificity alkaline proteases. Due to the unique cell wall composition of each plant species, the choice of enzyme can determine the protein yield recovered and, thus, this explains to some extent the wide range of protein yields reported to date in the literature. Initial experimentation is required to optimise the enzyme operating conditions during EAE; however, a range of statistical tools are available to aid this process. This allows relatively rapid identification of the most appropriate enzyme or enzyme combination (different proteinases, different carbohydrases and combinations of proteinases and carbohydrases), E:S, operating temperature, pH, duration of incubation, etc. Minimising, e.g., the concentration of enzyme used and reducing the reaction duration can facilitate the economic feasibility of EAE processing at an industrial scale. There is clear evidence that the application of EAE enhances protein recovery from different plant sources compared to other extraction processes. Interestingly, proteases represent the majority of the enzymes used to aid plant protein extraction. This may be linked to the enhancement of the techno- and bio-functional properties of the extracted proteins/peptides following EAE. However, the downstream application(s) and the purity of the protein isolate/protein hydrolysate needs to be considered when selecting a protease aided extraction approach as the proteins extracted can be converted to lower molecular mass protein molecules and peptides which alters their physicochemical, functional and bioactivity properties. While enzyme-based extraction processes offer a high protein recovery yield, the cost of enzyme also needs to be carefully evaluated when applying EAE at industrial scale. However, significant developments are taking

place in this regard with the development of enzyme membrane reactors which allow the reuse of enzymes while continuously processing the extracted proteins/peptides. Furthermore, the application of other novel processing approaches such as microwave, ultrasonic, high-pressure processing, etc., to aid cell wall disruption during biomass pre-treatment in conjunction with EAE represent significant promise in the development of sustainable green plant protein extraction processes.

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High Pressure for Plant Protein Extraction

7

Alice Marciniak and Alain Doyen

Abstract

In addition to improving shelf life while preserving the nutritional quality of food, high-pressure processing is gaining interest for the production, separation, and extraction of high-value compounds such as proteins. Through its effect on the matrix structure, high-pressure technology is improving penetration of extraction solvent within the cell and/or modifying the protein functionality such as their solubility. In this perspective, extraction assisted by high-pressure technologies is highly promising for the extraction of proteins from plants for food and/or pharmaceutical purposes. This book chapter will review the main aspects of high-pressure processing and summarize studies on the effect of pressure parameters on protein's extraction.

Keywords

High hydrostatic pressure · high-pressure homogenization · protein denaturation · compression · shear

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Abbreviation

HHP	high hydrostatic pressure
HPAE	high-pressure-assisted extraction
HPH	high-pressure homogenization
MPa	megapascal, 0.1 MPa = 1 bar

7.1 Introduction

Proteins are of high nutritional and economic interests and although widely present in food and nature, remain a highly valuable molecule (Martínez-Monteagudo et al. 2017). Their extraction from plant can be technically and environmentally challenging using conventional extraction methods. In addition, consumer's expectations have prompted the appearance of various emerging non-thermal technologies such as high-pressure-based processing in the food industry (Roobab et al. 2021). Firstly used for improving shelf life and thus improving food preservation, the application of high hydrostatic pressure (HHP) and high-pressure homogenization (HPH) is now widening up. Indeed, with their specific impact on macromolecules through pressurization phenomenon or combination of pressure, shear, cavitation, and turbulence, HHP and HHP have been demonstrated to be efficient in improving the extraction of protein from plant matrices (Balasubramaniam et al. 2016). This chapter will review the generalities of HHP and HPH and their impact on protein denaturation and summarize the current literature on their application for the extraction of proteins from plant, along with their advantages, disadvantages, and challenges.

7.2 High-Pressure Processing

7.2.1 High Hydrostatic Pressure

7.2.1.1 Generalities

High Hydrostatic Pressure (HHP), also known as "cold pasteurization" or pascalization, is mainly used in the food industry for the inactivation of spoilage and pathogen microorganisms. Its use dates back from the late 1800 for inactivating spoilage of milk with pressure of 685 MPa (Hite 1899). Despite its commercial use in Japan in the late 1990, this is only in 2009 that the Food and Drug Administration (FDA) approved the use of HHP for commercial pasteurization of low acidic foods (Yordanov and Angelova 2010). Typically, the process involves pressures ranging from 100 to 600 MPa for 0–30 min, coupled or not with heat. The phenomenon involved is based on three main principles, which are:

1. Isostatic principle states that any constraint applied to the surface of a fluid is uniformly transmitted. The effect of pressure is instantaneously and homogeneously distributed on every point of the products, regardless of their shape and size.
2. Le Chatelier's principle states that the equilibrium of a system will shift toward a new equilibrium when it is subjected to constraints (here pressure). This new equilibrium is along with a volume decrease (up to 15% decrease at 600 MPa) and temperature increase (3 °C/100 MPa).
3. Principle of microscopic ordering states that the increase of pressure induces an increase of the molecules' rearrangement degree.

Such pressure induces drastic compression of the system, which water is the major compound. The compression of water is responsible of the important effect of HHP on food components such as protein, while preserving heat-sensitive components such as vitamins (Balasubramaniam et al. 2015; Huppertz et al. 2019; Pereira and Vicente 2010). Up to today, HHP has been used commercially for the pasteurization of fresh products, such as fresh fruit juices and jams, guacamole, meat-based meals, etc. However, due to the ability of the process to induce specific structural changes in proteins and hence affect their functionality and bioactivity, studies are now focusing on innovative applications of HHP. Therefore, it represents a very promising technology for the extraction and purification of protein from different food matrices (Bermúdez-Aguirre and Barbosa-Cánovas 2011; Jermann et al. 2015; Pereira and Vicente 2010).

Two different types of HHP systems are available: direct and indirect (Fig. 7.1a). Direct pressurization system is mainly used in laboratory or pilot plant scales and is composed of a piston which compresses the pressure-transmitting fluid (usually a mix of glycol and water) up to the targeted pressure. In comparison, the pressure in indirect pressure system is built by pumping the pressure-transmitting fluid (usually

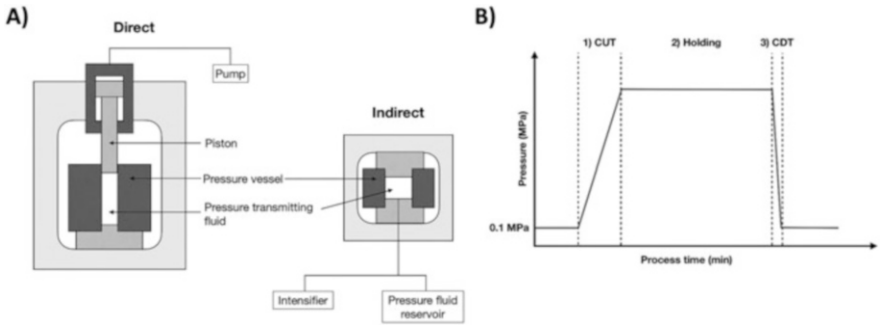

Fig. 7.1 Graphical illustrations of direct and indirect high hydrostatic pressure system (**a**) and diagram of typical high hydrostatic pressure cycle (**b**). CUT: come-up time, time to reach targeted-pressure; CDT: come-down time, time to release pressure up to atmospheric pressure (usually <1 s). Adapted from Yordanov and Angelova (2010); Balasubramaniam et al. (2016); Aganovic et al. (2021)

water) into the pressure vessel, up to reaching the targeted pressure (Yordanov and Angelova 2010). In a typical HHP cycle (Fig. 7.1b), the matrix is vacuum packed in flexible polymeric package and loaded into the pressure vessel (Fig. 7.1a). The vessel is then closed and filled with the pressure-transmitting fluid, which increases the pressure within the system (Fig. 7.1b—come-up time, CUT). Pressure is then held for desired time (Fig. 7.1b—Holding time) prior to an instantaneous decompression (Fig. 7.1b—come-down time, CDT) (Aganovic et al. 2021). Lastly, the processed product is unloaded from the system. Throughout the increase of pressure, the food matrix is subjected to an increase of temperature of around 3 °C/100 MPa, to eventually returning back to its initial temperature upon decompression (Balasubramaniam et al. 2016, 2015).

7.2.1.2 Thermodynamics of Proteins under High Pressure

Protein state is regulated by the elliptic phase diagram (Fig. 7.2a). Native proteins are stable up to a certain extent (blue ellipse). Any changes in terms of pressure and temperature can induce the denaturation of the proteins (Heremans and Smeller 1998). During HHP treatment, as a result of water volume decrease (Fig. 7.2a—$\Delta V < 0$), the water molecules are forced to enter inside the protein matrix, which induces structural transitions and modify the hydration degree to eventually result in the denaturation and aggregation of the proteins (Fig. 7.2a—denatured proteins) (Huppertz et al. 2019). The effects of HHP on protein have been largely documented in the literature and are summarized in Fig. 7.2b.

Protein conformation and thus stability and functionality are maintained by several non-covalent and covalent bonds which are differently impacted by HHP. Among the chemical bonds, hydrophobic and ionic, which are weak interactions, are characterized by the most negative constant of dissociation and thus are the most pressure sensitive. Their destabilization under HHP induces the dissociation of the specific native three-dimensional structure of the protein. On the other hand, hydrogen bonds are described to be stable up to 400 MPa and covalent bonds, such as disulfide and peptide bonds, are not disrupted (Rivalain et al. 2010). As a result, the

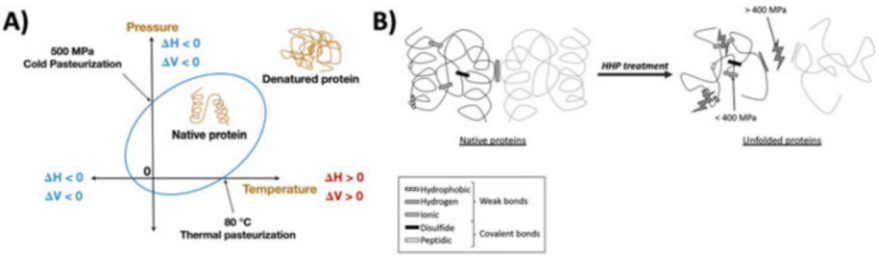

Fig. 7.2 Elliptic phase diagram of protein stability and microbial inactivation (black arrows) under high pressure and thermal treatments (**a**) and impact of high hydrostatic pressure on chemical bonds present in proteins (**b**). ΔH: change in enthalpy (<0: exothermal reaction, >0: endothermal reaction) and ΔV: change in volume. Adapted from Heremans and Smeller (1998); Barbosa-Cánovas and Lelieveld (2016); Marciniak et al. (2018)

primary and part of secondary structure of the protein are preserved under high pressure.

Consequently, the entry of water within the protein is forcing its unfolding and induces structural and conformational transitions that can be reversible or irreversible. These modifications of the physicochemical properties of the protein widen the possibility for their extraction assisted by high-pressure technology.

7.2.2 High-Pressure Homogenization

7.2.2.1 Generalities

Another high-pressure-based process has shown interesting effects on food matrices. Indeed, High-Pressure Homogenization (HPH), which is used for different purposes such as homogenization of fat, or food pasteurization and spore inactivation is available industrially (Dong et al. 2015). It is a pressure and shear-based technology that consist in forcing a fluid to pass through a valve, which induces pressurization of the fluid (up to 400 MPa) and size particle reduction (Balasubramaniam et al. 2015). As can be seen in Fig. 7.3a, typical HPH systems are composed of a pump, which brings the coarse fluid into the system, up to a restriction valve responsible for the pressure increase. Following the flow through the valve, the fluid is rapidly cool down when passing through the cooling coil. The fluid is pressurized through the valve, which closure (Fig. 7.3a—gap) is adjusted to reach targeted pressure. In order to enhance the impact of HPH on the matrix, the fluid can be recirculated into the system several times (number of pass) (Maresca et al. 2011).

During typical HPH treatment, when the fluid encountered the restriction valve, it is subjected to various phenomena as presented in Fig. 7.3b. Indeed, due to the restricted space (Fig. 7.3a—gap) at the entrance of the valve, the pressure of the fluid is increasing, and it induces high-hydrostatic pressure phenomenon. However, this phenomenon is observable for a very short time (<1 s) relatively to HHP technology.

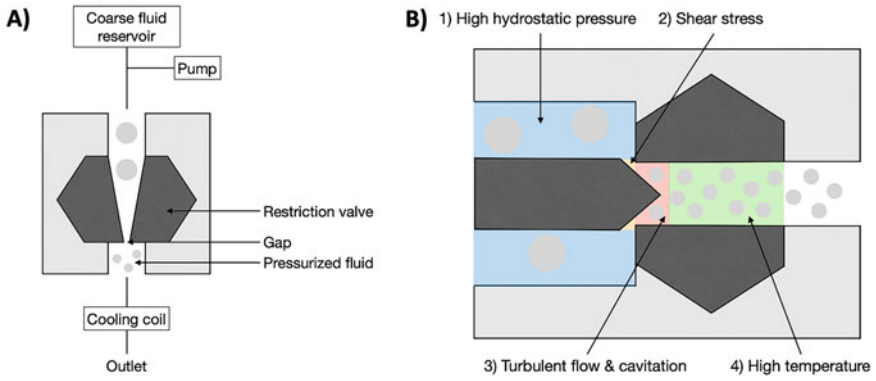

Fig. 7.3 Diagram of high-pressure homogenization system (**a**) and the simultaneous physical phenomena involved during the treatment (**b**). Adapted from Balasubramaniam et al. (2016)

Through the passage of the fluid through the gap, it is subjected to shear stress which is followed by an increase of the temperature in the system. This increase is irreversible and can reach approximately 18 °C/100 MPa in comparison with 3 °C/100 MPa for HHP. Lastly, due to the difference of pressure at the entrance and exit of the valve, the fluid is subjected to turbulent flow and cavitation phenomena, from which the energy is sufficient to enhance the breaking of the particle (Yong et al. 2021; Martínez-Monteagudo et al. 2017). Due to the combination of physical stresses applied on the food matrix, HPH induces structural changes in the food matrix, including proteins (Balasubramaniam et al. 2015).

7.2.2.2 Thermodynamics of Proteins under High Pressure

Contrary to HHP, the general effect of HPH on proteins is poorly reported due to the complexity of the phenomena involved. In addition, the effect is highly dependent on the matrices investigated. Overall, the effect of pressure itself is much lower than the one observed in HHP due to shorter times under pressure (<1 s) (Balasubramaniam et al. 2016). In addition, the presence of a cooling coil immediately after the pressurization valve also diminishes the impact of temperature on the protein's denaturation leading to few impacts on secondary and tertiary structure of proteins for pressure up to 400 MPa, with very low formation of disulfide bonds under HPH treatment (Balasubramaniam et al. 2016). In contrast, quaternary structure seems to be impacted by the treatment up to a certain extent. Indeed, due to the shear forces, weak chemical bonds such as hydrophobic interactions are responsible for the stability of the protein and can be disrupted for pressure up to 250 MPa. However, for pressures above 250 MPa, an important increase of temperature is induced, which enhances the stability of those bonds and diminishes the impact of HPH (Wang et al. 2020).

7.3 High-Pressure Assisted Extraction

7.3.1 Generalities

High-pressure technologies are gaining a lot of interest as green technologies for improving the extraction of bioactive compounds, including proteins. Due to the phenomenon involved in pressure-based processing, the efficiency of high-pressure assisted extraction (HPAE) drastically relies on pressure and time level for HHP, and number of pass for HPH, as well as the nature of the solvent used (Balasubramaniam et al. 2016; Rodriguez-Gonzalez et al. 2015). As the role of solvent has been widely covered in the previous chapter, this chapter will focus on the pressure parameters and their impact on protein extraction from various plant sources.

In the last 20 years, HPAE has been studied on different matrices such as rice (grain and bran), soybean, oat, and pollen, but also on several macro- and microalgae (Table 7.1). HPAE of proteins by HHP is usually achieved according to two protocols, as described in Fig. 7.4a and b. The matrix can be either treated under pressure with water (Fig. 7.4a—two-stage extraction) or with a specific extraction

Table 7.1 High-pressure (hydrostatic and homogenization)-assisted extraction of plants (adapted from Kumar et al. (2021))

Source	Parameters	Results	Reference
High hydrostatic pressure:			
Rice grain	400 MPa—30 min 300 MPa—120 min	3.5 times higher recovery 5 times higher recovery	Kato et al. (2000)
Rice bran	500 MPa—10 min	3% increase of protein recovery when combined with amylase	Tang et al. (2002)
Soybean flakes	200 MPa—15 min 500 MPa—15 min	22.5% (200 MPa) and 23.6% (500 MPa) increase of protein solubility coupled with enzyme (Protex 7 L)	Jung and Mahfuz (2009)
Oat batter	300–500 MPa—10 min	2 times decrease of soluble protein in urea buffer	Hüttner et al. (2009)
Pollen (*Cedrus atlantica*)	330 MPa—30 min	13 times higher recovery yield	Altuner et al. (2012)
Soleria chordalis	400 MPa—20 min	8.4% increase of recovery yield 17% increase when coupled with cellulase	Suwal et al. (2019)
Palmaria Palmata	400 MPa—20 min	5% increase of recovery yield when coupled with hemicellulase	Suwal et al. (2019)
High-pressure homogenization:			
Peanut	80 MPa—1 pass	23% increase of recovery yield	Dong et al. (2011)
Chlorella vulgarisI	270 MPa—2 passes	98% recovery yield	Ursu et al. (2014)
Soybean slurry Soybean okara	100 MPa—1 pass 100 MPa—1 pass	20% increase of protein recovery yield 25% increase of protein recovery yield	Preece et al. (2017)
Chlorella vulgaris	150 MPa—5 passes	200 times higher recovery yield	Carullo et al. (2018)
Soybean okara	150 MPa—1 pass 150 MPa—5 passes	55% increase of protein recovery yield 78% increase of protein recovery yield	Fayaz et al. (2019)
Parachlorella kessleri	120 MPa—1 pass/ 10 passes	3 times increase of protein recovery yield from 1 to 10 pass	Zhang et al. (2019)

solvent (Fig. 7.4b—single-stage extraction). In the two-stage extraction, the sample is suspended in water (1) and pressurized for a targeted time and pressure inducing cell damage and deformation (2). Then the pressure-treated matrix is re-suspended into a specific solvent, to extract compounds of interest (3). This step can be coupled with a mechanic treatment to induce a breakage of the cell and increase the efficiency of the extraction. Regarding the single-stage extraction (Fig. 7.4b), the matrix is directly suspended into the solvent of extraction (1) and pressurized for a targeted time and pressure (2). This results into an ultrastructural modification which is responsible for higher permeability of the membrane and thus penetration of the

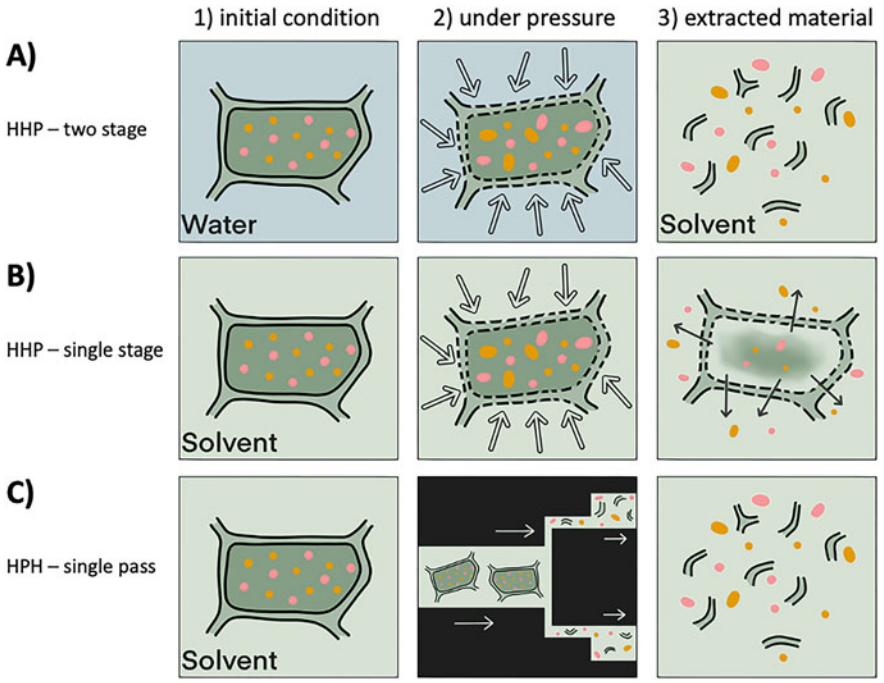

Fig. 7.4 Representation of high-pressure-assisted extraction using high hydrostatic pressure (HHP) two stage (**a**), and single stage (**b**) and high-pressure homogenization (**c**). Adapted from Balasubramaniam et al. (2016); Huang et al. (2020); Olmstead et al. (2013)

solvent into the plant cell (2) to eventually increase the solubility of compounds of interests, and their release in the extracellular phase (3). Lastly, HPAE of proteins is also possible using HPH treatment (Fig. 7.4c). In this scenario, the matrix is directly suspended into the extraction solvent (1), and the phenomena involved during processing induce harsh breakage of the cells (2) to eventually results in the release of the targeted material in the solvent (3).

7.3.2 Pressure Assisted Extraction of Proteins

Overall, literature shows that higher levels of pressure are correlated with an increase in protein extraction. For instance, Altuner et al. (2012) were able to increase the extracted protein's concentration (from pollen) from 1.95 mg/mL with conventional extraction method, to 18.02 mg/mL with HHP treatment of 220 MPa—10 min and up to 23.38 mg/mL at 330 MPa—10 min. Similarly, a study on rice grain conducted by Kato et al. (2000) observed that an increase of the pressure from 0.1 MPa to 100 MPa—30 min and ultimately 400 MPa—30 min, enhanced the protein extraction from 0.1 mg/g of rice to 0.23 mg/g (100 MPa) and 0.35 mg/g (400 MPa), respectively. In contrast to those studies, Hüttner et al. (2009) observed a lower

protein extraction yield from oat batter when pressure was increased from 200 to 500 MPa. For instance, the authors were able to recover close to 40 mg/g of soluble protein when untreated, against 30 mg/g at 300 MPa and less than 20 mg/g at 500 MPa. These results were potentially explained by the interactions between proteins and other compounds present under pressure.

In the same way, time under pressure plays a major role in the efficiency of protein extraction from plants. Indeed, a longer time under pressure allows an increase in the contact time between the solvent and the compounds of interest (Kumar et al. 2021). Kato et al. (2000) demonstrated that increasing the pressure treatment time from 10 to 120 min allows increasing the protein recovery from rice grain by two times. Likewise, Altuner et al. (2012) measured an increase of the soluble protein concentration when pollen was HHP-treated at 220 MPa for 30 min in comparison with 10 min (22.57 mg/mL and 18.02 mg/mL).

Another way of increasing the efficiency of the HPAE is the coupling of pressure with enzymatic hydrolysis. Indeed, when pressurization and enzymatic hydrolysis are simultaneous, the reaction can be either inactivated or enhanced (Marciniak et al. 2018). By specifically choosing pressure and time parameters as well as the enzyme, the extraction can be improved by inducing (1) plant cell breakage and (2) higher solubility of the proteins through their unfolding and/or hydrolysis. Studies performed on rice bran (Tang et al. 2002), soybean flakes (Jung and Mahfuz 2009), and macroalgae (*Soleria chordalis*). Suwal et al. 2019 have demonstrated an increase in protein recovery when enzymatic hydrolysis was coupled with pressure in comparison with high pressure alone. For instance, Jung and Mahfuz (2009) were able to increase soybean flakes protein solubility, and thus their recovery in the solvent by up to 23.6% when pressure (500 MPa—15 min) and enzyme were combined, in comparison with pressure alone. Those observations can be explained by the formation of smaller polypeptides and/or the structural changes due to the action of the protease. However, these effects were significant only for high pressure, since pressurization at 200 MPa did not increase the protein yield (Jung and Mahfuz 2009).

The effect of the pressure treatment duration, and of the level of pressure on the protein extraction from plant can be explained by a good understanding of the phenomenon involved during the treatment. As HHP is following the isostatic principle, the pressure is uniformly applied on the products independently of its mass, shape, and composition. As a response to that phenomenon, molecules and compounds are reducing their volume, which affect their structure and potentially their functionality such as solubility (Kumar et al. 2021; Huang et al. 2020). In the case of raw matrices such as micro/microalgae, the cell wall, which is composed of polysaccharides, is more resistant, whereas the cell membrane, composed of lipids and proteins, is more sensitive to high pressure. Thus, the coupling with enzymes (pectinase or carbohydrase) to improve the permeability of the wall and membrane becomes very interesting. With the increase of the pressure treatment duration, the matrix is reaching a new equilibrium between the inside and outside of the cell, improving the penetration of the extraction solvent through the cell and thus the contact time with the proteins (Huang et al. 2013, 2020). Finally following

instantaneous decompression, the cell expands increasing the damage and resulting into the release of the proteins from the cytosolic medium into the extracellular solvent (Kumar et al. 2021; Huang et al. 2013).

Similarly to HHP, literature on the effect of HPH on protein extraction from plants have shown that pressure parameters (pressure level and number of pass) have a major role in the extent of the extraction. Research has been conducted on different matrices such as peanut, soybean (slurry and okara), and microalgae (*Chlorella vulgaris* and *Parachlorella kessleri*).

Overall, the level of pressure during HPH was described to increase the protein recovery in comparison to conventional extraction. Indeed as observed by Dong et al. (2011) HPH increases protein extraction yields from peanut by 13.8% at 40 MPa and up to 23% at 80 MPa. Similarly, Preece et al. (2017) demonstrated that the use of pressure was correlated with higher protein extraction yield, up to a certain extent. As a matter of fact, when applying HPH from 0 to 120 MPa, on soy slurry and soy okara, the authors showed that the extraction yield was increased by up to 18% and 5% at 60 MPa (respectively for the soy slurry and soy okara), whereas pressure up to 120 MPa did not lead to higher yield. In contrast to previous authors, Fayaz et al. (2019) were able to increase the protein extraction yield from soy okara by 26% at 50 MPa and by 5% at 150 MPa. To a higher extent, a study conducted by Ursu et al. (2014) on a suspension of *Chlorella vulgaris* highlights that the use of HPH at 270 MPa increases the protein recovery to reach 98% in comparison with 4.3% with conventional extraction.

Another parameter that has been deeply studied for its effect on the protein extraction yield is the number of passes within the system. Indeed, by multiplying the number of passes, the matrix is subjected to higher mechanical stress that can therefore impact the protein extraction yield. Contradictory results have been observed regarding the impact of number of pass on protein extraction yield. For instance, Preece et al. (2017) observed a decrease in the protein extraction yield when number of pass was increasing to eventually reach similar yield as non-pressurized samples (100 MPa from 1 to 5 passes—soy slurry). In contrast, Ursu et al. (2014) observed an increase of soluble protein by around 15% when the number of pass increased from 1 to 2 (270 MPa—*Chlorella vulgaris*). Likewise, Carullo et al. (2018) showed that HPH treatment of 150 MPa—1 pass was able to increase the soluble protein concentration up to 95 times, while 5 passes led to 200 times higher recovery. Similarly, Fayaz et al. (2019) observed a 23.75% increase of protein extraction yield when soy okara was pressure-treated at 150 MPa—5 passes, in comparison with 1 pass. Lastly, Zhang et al. (2019) observed a similar impact of the number of passes on protein extraction from *Parachlorella kessleri* but to a certain extent. They observed noticeable increase of the degree of extraction up to 4 passes with no increase above 4 passes (120 MPa).

The effect of pressure level and number of pass on the protein extraction yield can be explained by an increase of the contact surface area exposed to the solvent of extraction with the shear and pressure phenomenon involved during HPH (Dong et al. 2011). In addition, literature mostly reports that the main effect on protein extraction is happening after 1 pass. Indeed, the first HPH pass might damage and

disrupt the cell wall/structure of the matrix and thus increasing the solubility and/or access of the solvent to the targeted compounds (proteins). The effect of additional passes remains controversial and highly dependent on the matrix (Carullo et al. 2018).

While HPAE globally results in improved protein extraction, it also improved proteins functionality through the modification of the secondary structure and thus improved the final quality of the extract. A recent review highlights that HHP on plant proteins globally improves the water holding capacity, solubility, gelation, emulsion, and foaming properties as well as protein digestibility while decreasing their allergenicity. Similar observations have been demonstrated following HPH treatment. However, similarly to the protein recovery, these results are highly dependent on the type and source of protein, as well as the extraction parameters (Gharibzahedi and Smith 2020; Saricaoglu 2020; Shkolnikov et al. 2021).

7.4 Pros and Cons of Pressure-Based Processes

Both HHP and HPH systems are already used at industrial level mainly for the pasteurization and sterilization of food matrices. In addition to the difference in operating parameters (summarized in Table 7.2), they show different features and pros and cons in terms of operation, maintenance, cleaning, and energy requirements.

The main difference between the two processes relies on the operation mode of the system. Indeed, while HPH is a continuous system and allows treatment of a large quantity of product, HHP is in contrary a batch system and its productivity depends on the volume of the vessel. However, this impacts the range of product that can be treated. As a matter of fact, the use of HPH is limited to liquid matrices, while HHP can be applied to a wider range of products. In addition, in a context of HPAE, HHP possesses the advantage to use packaged products in comparison with bulk

Table 7.2 Comparison between high hydrostatic pressure (HHP) and high-pressure homogenization (HPH). Adapted from Martínez-Monteagudo et al. (2017)

Parameters	High Hydrostatic Pressure (HHP)	High-Pressure Homogenization (HPH)
Mode of pressure application	Hydrostatic	Discharge through valve
Principle	Reduction of intermolecular distance	Dissipation of kinetic energy
Pressure level	Up to 600 MPa	Up to 350 MPa
Temperature rise	3–8 °C/100 MPa—reversible	15–18 °C/100 MPa—irreversible
Application	Food preservation	Particle size reduction, emulsification, and food preservation

fluid for HPH system. Thus, it is drastically decreasing the environmental impact of the cleaning step of HHP since water is the only effluent discarded and can also be reintroduced into the system to minimize the operating cost but also energy loss (Pereira and Vicente 2010; Balasubramaniam et al. 2015). In contrary, HPH requires a chemical cleaning procedure, which generates chemical wastes that need to be treated. In addition, the energy consumption per food product unit of HHP system considerably depends on the vessel filling which must be maximized (Pereira and Vicente 2010). Indeed, it has been demonstrated that with 20% of vessel filling efficiency, the electric power was reaching close to 1000 kWh/MT in comparison to 200 kWh/MT when vessel was filled at 80%. Overall operating costs represent 20–25% of the total cost (Pereira and Vicente 2010; Rodriguez-Gonzalez et al. 2015; Toepfl et al. 2006). HHP systems are already used industrially for commercial use and world widely implemented. However, due to energy and cost requirements, their use is limited to high-value-added products. On the other hand, HPH's commercial application is still under development. Indeed, due to engineering challenges, there are still important developments required to develop industrial-scale systems. Among these improvements, research have intensively focused on the valve geometry and configuration in order to improve the energy dissipation within the system (Martínez-Monteagudo et al. 2017).

Due to their specificity, specifically the non-use of heat, HP-based processes are of high interest to maintain valuable compounds' activity, including proteins. In addition, the technologies are still emergent and continuously developing, leading to improved units, with better control of the parameters and wider applications. In comparison with conventional extraction, HPAE is very promising since it can improve the extraction of proteins while decreasing the use of solvent, the time of extraction as well as avoiding the use of heat, but also improve the quality of the protein extract. Thus, HPAE represents an eco-friendly alternative to conventional extraction (Kumar et al. 2021).

7.5 Conclusion

Over the last decades, high-pressure-based technologies have shown growing industrial interest. However, due to their recent emergence, their operational, technical, and environmental challenges, their application remains limited to high-value-added products. In that order, HHP and HPH are promising technologies for the HPAE of proteins from plant sources. Indeed, their application on the matrices can induce cell breakage, increase membrane permeability and protein solubility in the solvent of extraction. In addition to the solvent of extraction, the efficiency relies also on the choice of parameters such as the initial temperature, pressure level, time of treatment or number of passes. However, to widen their industrial use and thus reduce the total cost of the process, efforts are still necessary to counteract the technical and environmental challenges that face these processes.

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High Voltage Electrical Treatments as an Eco-Efficient Approach for Plant Proteins Processing

8

Sergey Mikhaylin

Abstract

The current issues regarding food safety and at the same time food losses and waste along with constantly increasing environmental impacts need the eco-efficient solutions from the modern agri-food industries. Thus, this chapter will present the emergent High Voltage Electrical Treatments (HVET) including Pulsed Electric Field and Arc modes as a technology allowing to tackle the above-mentioned issues. Indeed, HVET allow to improve extraction and bio-functional properties of food molecules with the minimal environmental impact comparing to conventional technologies. The focus will be on the protein-rich food matrices. Firstly, the HVET principles and mechanisms of action will be discussed. Then, the conventional and emergent applications of HVET to produce the high added-value functional and bioactive proteins and peptides will be presented. Finally, the main limitations and future prospects of HVET technology will be highlighted.

Keywords

Eco-efficiency · High voltage electrical treatments · Plant proteins · Peptides · Food waste

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8.1 Introduction

The multiple scientific innovations and technological progress allowed to human population a very rapid development and growth, which accelerated remarkably from the beginning of the twentieth century. Indeed, nowadays the global population accounts for 7.9 billion people compared to 1.6 billion in 1900. Moreover, the population is expected to attain 9.7 billion in 2050 and 10.9 billion in 2100 (United Nations 2019). Along with the positive changes (e.g., economic growth, improved quality of life and literacy), multiple issues arose such as growth of inequalities between rich and poor people, intensive resource consumption, and high environmental impacts. Looking at the agri-food system, one can notice significant technological progress allowing to increase substantially the agricultural production and food security (FAO 2017). However, often the gains in productivity are accompanied by negative impacts on the environment (e.g., loss of biodiversity, land degradation, water scarcity, pollutions). Moreover, 1.3 billion people still have important issues with the regular access to nutritious and sufficient food, while about one-third of the globally produced food is lost or wasted every year (FAO and WHO 2019; FAO 2019). These facts along with the increase in food demand (50% increase by 2050) raise serious challenges for the agri-food system. To address this unprecedented population growth and current challenges, the modern agri-food system should considerably increase its productivity in sustainable way. One of the approaches allowing to tackle the above-mentioned issues is to involve the eco-efficiency concept in the product development, meaning to increase the value of the product or service while reducing its environmental impacts (ISO 2012). Initially, the economical value (e.g., profit, cost, willingness to pay, unit of product or service) was considered in the eco-efficiency concept. However, the ISO 14045 defined other possible values, which can be considered in eco-efficiency assessment, such as functional properties, desirability, nutritional value, esthetic, cultural, and historical values. Regarding the environmental aspect of eco-efficiency, it is recommended to use a life cycle assessment in accordance with ISO 14040 and 14,044 to determine the environmental burden associated with the product or service system. The environmental impact can be expressed as water, energy, or resource consumption, green house gas emissions, ecosystem quality, human health, etc., depending on the goal and scope of study. It is worth noting that eco-efficiency is a relative concept meaning that the product system can be more-or-less eco-efficient only in relation to another product system. Thus, the current chapter will focus on plant-based proteins, which represent one of the most important pillars of modern sustainable diets (FAO and WHO 2019), providing human society with healthy and nutritious food with a minimal environmental impact. More specifically, the emergent High Voltage Electrical Treatments (HVET) will be considered in order to tackle eco-efficient production and processing of proteins from plants. Indeed, according to the North American and European experts, HVET are considered among the most promising emergent technologies for industrial commercialization aiming at increasing the value of the wide array of foods with minimal energy requirements (Jermann et al. 2015; Barba et al. 2015).

8.2 Principles of High Voltage Electrical Treatments

High Voltage Electrical Treatments (HVET) consist in the application of high electric field strength (0.1–80 kV/cm) in pulse mode (ns-ms) to treated food matrix (Miklavcic 2019). The aim of such high voltage is to induce desirable changes in the level of cells and food molecules (e.g., proteins, carbohydrates). Two main HVET modes exist, namely Pulsed Electric Field (PEF) and electric arc or High Voltage Electrical Discharge (HVED) (Fig. 8.1). In the PEF mode, food treatment occurs between two plate electrodes while at arc mode the treated product is placed between plate cathode and needle anode. These two modes of HVET differ by phenomena occurring during the application of high voltages. Indeed, during operation at PEF mode, there is a generation of electromechanical stress and chemically active species (e.g., O_3, H_2O_2, •OH, •OOH, $O_2^{\bullet -}$), while at electrical arc mode the additional phenomena take places such as the formation of shock waves, vapor cavities, and UV-light (Boussetta et al. 2013; Pataro et al. 2016; Bryant and Wolfe 1987).

HVET treatments have demonstrated high efficiency in multiple food processes (inactivation of microorganisms, improvement of drying, freezing, osmotic dehydration, extraction, etc.) (Barba et al. 2015; Vorobiev and Lebovka 2020). There are more than 100 industrial installations for the PEF equipment dedicated to the improvement of juice extraction from sugar beets and oil extraction from olives, improvement of cutting and peeling performance during French fries and snacks processing, extension of fruit and vegetable juice shelf life, etc. (Barba et al. 2020;

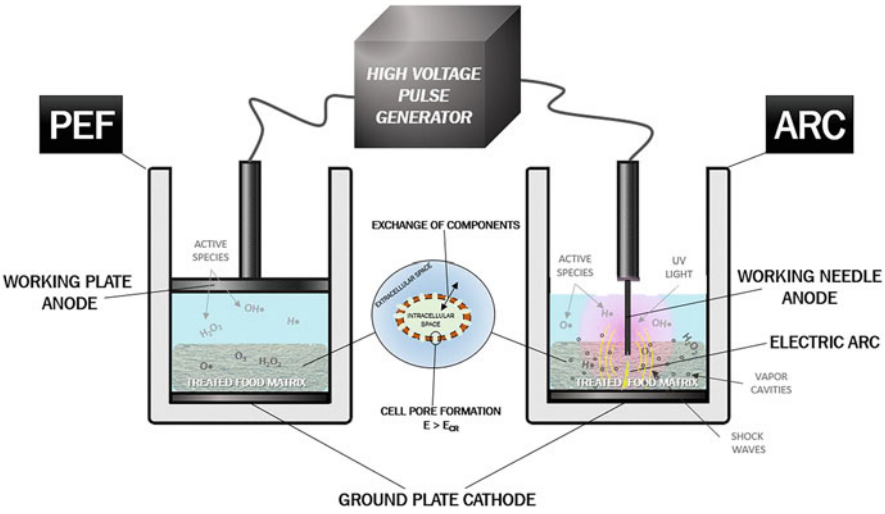

Fig. 8.1 The schematic representation of the main components of High Voltage Electrical Treatments (HVET) and electroporation phenomenon. PEF refers to Pulsed Electric Field mode and Arc refers to Electric Arc (or High Voltage Electrical Discharge) mode. E and E_{CR} are the applied electric field strength and critical electric field strength at which electroporation occurs, respectively

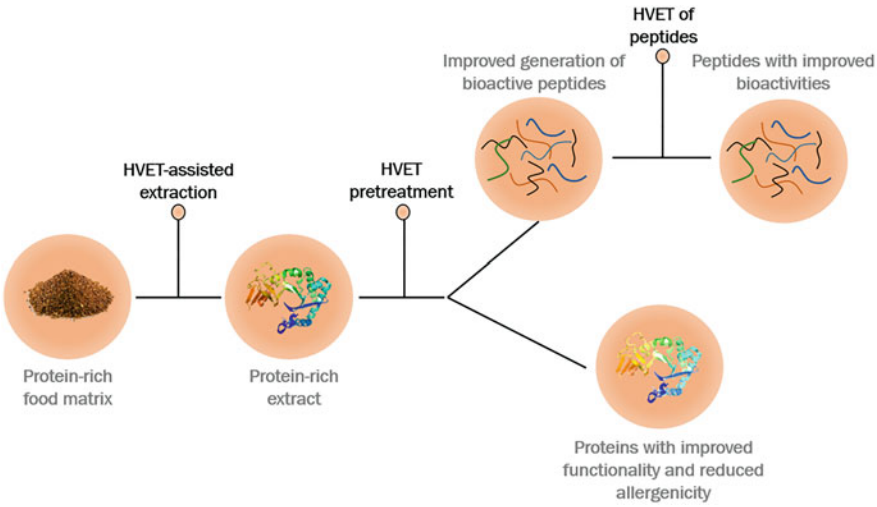

Fig. 8.2 Scheme of potential HVET applications to protein-rich food matrices and peptides

Vorobiev and Lebovka 2020). However, arc (or HVED) treatments are still being explored at the laboratory and pilot scales. Moreover, there is no equipment allowing the continuous processing of foods for arc mode while such an equipment is available for PEF mode. The continuous mode is used for the pumpable liquid foods (e.g., juices, milk, liquid eggs), whereas the batch mode is suitable for the solid and semi-solid foods. However, it is worth noting that solid foods can be treated in continuous mode using the belt systems allowing the processed product to be transported between two parallel plate electrodes in a water bath (Vorobiev and Lebovka 2020; Kempkes 2017).

The main parameters, which influence the treatment efficiency, are the following:

- Electric field strength
- Pulse duration
- Number of pulses and their frequency
- Pulse shape (exponential or rectangular)
- Pulse polarity (monopolar or bipolar)
- Properties of the treated food matrix (conductivity, pH, temperature)
- Design of treatment chamber
- Hydrodynamic conditions for continuous operation

The conventional direction of application of HVET comprises the electroporation phenomenon illustrated in Fig. 8.1. This phenomenon occurs in the cells (e.g., bacterial, yeast, plant, or animal tissues) when applied electric field strength is higher than the critical value of cell membrane potential difference (~1 V). The critical electric field strength strongly depends on the cell size, structure, concentration, and geometry as well as the properties of the treated media (Barba et al. 2015; Tylewicz

2020). For instance, the critical electric field strength for the large plant cells is of the order of 0.1–2 kV/cm, whereas the smaller microbial cells have higher values of the order of 3–14 kV/cm (Tylewicz 2020; Barba et al. 2015). It is worth noting that the electroporation phenomenon can be reversible and irreversible depending on the applied electric field strength and treatment duration. While the effects of HVET on the electroporation phenomenon are widely studied, their effects on food molecules such as proteins, carbohydrates, and others in relation to their structure, functionality, bioactivity, etc., remain an emergent direction. Moreover, the studies regarding arc mode are scarce compared to the PEF ones. The recent review of Giteru et al. (Giteru et al. 2018) discussed the studies considering the impact of PEF on the physicochemical properties of the proteins, carbohydrates, and their complexes. Additionally, the review and book of Barba et al. (2020, 2015) describe the effect of PEF on food polyphenols, vitamins, isoprenoid compounds, fatty acids, amino acids, pesticides, and toxins. The current chapter will focus on the impacts of HVET on proteins and especially on plant-based proteins. The different possibilities of applications of HVET to improve extraction of proteins, modification of their properties and generation of peptides are resumed in Fig. 8.2.

8.3 HVET-Assisted Extraction for Protein-Rich Extract Production

The most studied application of HVET in relation to plant proteins is their extraction from different food matrices (Table 8.1). The extraction improvement is based on the above-mentioned phenomenon of electroporation (Fig. 8.1), leading to significant improvement of extraction yields (Vorobiev and Lebovka 2020). Indeed, during the formation of pores in the cell membranes, the diffusivity of targeted protein molecules from the intracellular space towards the extracellular liquid is significantly improved (Vorobiev and Lebovka 2017).

The HVET pretreatment prior to extraction of proteins from sesame cake was reported to increase the diffusivity of proteins at two tested temperatures (20 and 40 °C) and the more substantial impact was observed for the electrical arc pretreatment (Sarkis et al. 2015). Overall, the application of HVET with the average energy input of 83 kJ/kg significantly reduced the extraction duration to attain a similar protein concentration in the final extract compared to unpretreated sample. Indeed, the protein diffusivity from sesame cake at 20 °C was 3 and 6 times higher in PEF and arc pretreated samples, respectively, compared to control sample, which explains the faster extraction of proteins from HVET-pretreated samples. The arc-assisted extraction of proteins at the energy input of 240 kJ/kg and liquid-to-solid ratio of 20 was significantly improved from rapeseed (9.41 g/100 g) and rapeseed press-cake (15.8 g/100 g) while unpretreated rapeseed extracts contained no proteins and about 8 g/100 g of proteins were recovered from unpretreated rapeseed press-cake (Barba et al. 2015; Vorobiev and Lebovka 2020). Yu et al. (2016, 2015) have demonstrated a high potential of PEF pretreatments to improve the protein extraction from rapeseed biomass. Indeed, these authors reported 80%

Table 8.1 HVET-assisted treatment applied to plant protein food matrices

Product	HVET experimental conditions	Reference
Protein extraction		
Sesame cake	E_{PEF} = 13.3 kV/cm, E_{ARC} = 80 kV/cm, t_{pulse} = 10 μs, f = 0.5 Hz	(Sarkis et al. 2015)
Rapeseed and rapeseed cake	E_{ARC} = 80 kV/cm, t_{pulse} = 10 μs, f = 0.5 Hz	(Barba et al. 2015, Vorobiev and Lebovka 2020)
Rapeseed biomass	E_{PEF} = 0.2–20 kV/cm, t_{pulse} = 10 μs, f = 0.5 Hz	(Yu et al. 2015) (Yu et al. 2016)
Olive kernel	E_{PEF} = 13.3 kV/cm, E_{ARC} = 80 kV/cm, t_{pulse} = 10 μs, f = 0.5 Hz	(Roselló-Soto et al. 2015)
Papaya seeds Vine shoots	E_{PEF} = 13.3 kV/cm, E_{ARC} = 40 kV/cm, t_{pulse} = 8.3 μs, f = 0.5 Hz E_{PEF} = 13.3 kV/cm, E_{ARC} = 80 kV/cm, t_{pulse} = 10 μs, f = 0.5 Hz	(Parniakov et al. 2015) (Rajha et al. 2014)
Mushrooms	E_{PEF} = 0.8–1.3 kV/cm, t_{pulse} = 1000 μs, f = 0.2 Hz	(Parniakov et al. 2014)
	$E_{PEF(optimal)}$ = 38.4 kV/cm, t_{pulse} = 2 μs, f = 400 and 800 Hz	(Xue and Farid 2015)
Athrospira platensis and Athrospira maxima	E_{PEF} = 30–40 kV/cm, t_{pulse} = 1–32 μs, f = 2–300 Hz	(Jaeschke et al. 2019, Käferböck et al. 2020, Akaberi et al. 2020)
Microalgae	E_{PEF} = 0.5–20 kV/cm, t_{pulse} = 2–2000 μs, f = 0.1–924 Hz	(Coustets et al. 2013, Lam et al. 2017, Lam et al. 2017, Buchmann et al. 2019, Parniakov et al. 2015, Gateau et al. 2021, Coustets et al. 2015)
Macroalgae	E_{PEF} = 1–7 kV/cm, t_{pulse} = 4–50 μs, f = 0.5–3 Hz	(Robin et al. 2018, Polikovsky et al. 2016, Prabhu et al. 2019)
Escherichia coli	E = 7.5-20 kV/cm, t_{pulse} = 1–2000 μs, f = 2–1000 Hz	(Martínez et al. 2020, Meglic et al. 2015, Ohshima and Sato 2004)
Yeasts	E = 2.5-50 kV/cm, t_{pulse} = 2–800 μs, f = 0.5–3000 Hz	(Ganeva et al. 2020, Martínez et al. 2016, Ohshima et al. 1995, Liu et al. 2012)
Protein functionality		
Enzymes	E_{PEF} = 4–50 kV/cm, t_{pulse} = 1–20 μs, f = 15–1500 Hz	(Zhao et al. 2012, Zhang et al. 2021, Li et al. 2008, Aguilo-Aguayo et al. 2008, Aguiló-Aguayo et al. 2008, Ohshima et al. 2007, Riener et al. 2008, Yeom et al. 1999)
Soy protein	E_{PEF} = 20–40 kV/cm, t_{pulse} = 2 μs, f = 500 Hz	(Li et al. 2007)

(continued)

Table 8.1 (continued)

Product	HVET experimental conditions	Reference
Canola protein	E_{PEF} = 5–35 kV/cm, t_{pulse} = 1–10 μs, f = 600 Hz	(Zhang et al. 2017)
α-Amylase-pectin complexes	E_{PEF} = 5–20 kV/cm, t_{pulse} = 1000 μs, f = 1000 Hz	(Jin et al. 2020)
Ara h 2, 6 (peanut 2S albumins), apple Mal d 1 and Mal d 3 proteins	E_{PEF} = 5–35 kV/cm, f = 2 Hz E_{PEF} = 0.05 V/nm, f = 2450 MHz	(Johnson et al. 2010) (Vanga et al. 2015)
Pru p 3 peach protein	E_{PEF} = 25 kV/cm, t_{pulse} = 3 μs, f = 1 Hz	(Tobajas et al. 2020)
Peptide production and bioactivity		
Algal biomass	E_{PEF} = 40 kV/cm, t_{pulse} = 1 μs, f = 1.5–3 Hz	(Akaberi et al. 2019)
b-lactoglobulin	E_{PEF} = 20 kV/cm, E_{ARC} = 80 kV/cm t_{pulse} = 10 μs, f = 0.5–1 Hz	(Mikhaylin et al. 2017, Agoua et al. 2020)
Soybean and pine nut peptides	E_{PEF} = 5–15 kV/cm, t_{pulse} = 13 μs, f = 1800–2400 Hz	(Zhang et al. 2021, Zhang et al. 2019)
Peptides in a mixed orange juice and milk beverage	E_{PEF} = 15–40 kV/cm, t_{pulse} = 2.5 μs	(Rivas et al. 2007)

E electric field strength, t_{pulse} pulse width, *f* pulse frequency

protein extraction yield from rapeseed leaves when applying PEF of 160 kJ/kg energy input compared to the unpretreated sample (20% of protein yield). The significant improvement of the protein extraction from the olive kernel was reported by Rosello-Soto et al. (Roselló-Soto et al. 2015) when applying arc pretreatment (~225 mg/L at 55 kJ/kg energy input compared to 115 mg/L for unpretreated sample). Moreover, the protein extraction efficiency of ultrasound was similar to the arc one, while PEF pretreatment had no impact indicating that specific additional phenomena occurring during arc pretreatment (e.g., shock waves, cavitation bubbles; Fig. 8.1) play an important role in electroporation of olive kernel cells. Rajha et al. (2014) have demonstrated the better performance of arc pretreatments compared to PEF in the protein extraction from vine shoots. Indeed, the maximum protein yields were 4.2 mg/g and 3 mg/g for arc and for PEF at ~254 kJ/kg, respectively, compared to 2 mg/g for unpretreated sample. Moreover, in the case of vine shoots, the ultrasound pretreatment was less effective than arc pretreatment and similar to the PEF one. The aqueous extraction of proteins from papaya seeds when assisted by HVET showed a substantial improvement compared to conventional extraction (Parniakov et al. 2015). The highest extraction efficiency at pH = 7

was attained when using arc pretreatment (~350 mg_{BSA}/L) followed by the PEF one (~45 mg_{BSA}/L) compared to the optimal conventional extraction at pH = 11 (~17 mg_{BSA}/L). This fact means that HVET can help to improve the final value of the extract (protein concentration) while decreasing the environmental impact of the product by avoidance of the use of chemical agent (alkali) meaning a substantial improvement of extraction eco-efficiency. The synergistic pressure and PEF-assisted extraction from mushrooms (*Agaricus bisporus*) demonstrated higher protein extraction efficiency (6 mg/g) compared to pressure extraction alone (3.5 mg/g) and similar protein yield compared to water-ethanol extraction (Parniakov et al. 2014). However, water-ethanol extracts demonstrated low colloidal stability, which was not the case of PEF-pressure assisted extraction producing extracts of better quality in terms of colloidal stability. Xue and Farid (2015) demonstrated that the yield of proteins in the extracts obtained by PEF-assisted extraction from *Agaricus bisporus* at 20 °C inlet temperature was significantly higher (48.92%) compared to conventional water extraction at 2.6 min (7.45%) and comparable when the conventional extraction lasted 60 min (44.75%). These authors reported the synergistic effect of electric field and temperature generated during PEF treatment in the improvement of protein yield during its extraction. Therefore, the use of short PEF treatments seems to be promising to obtain high protein yields from mushrooms avoiding the use of long-duration heat treatments, which are generally considered as high consumers of energy leading to the significant environmental impact. Moreover, long heat treatment can negatively affect the structure and functionality of extracted proteins. Thus, the PEF-assisted extraction seems to be more eco-efficient compared to conventional heat extraction.

The very promising direction of HVET application to assist protein extraction was reported on algal matrices. Several authors reported significant improvement of protein release from *Athrospira platensis* and *maxima* when the extraction was assisted by PEF (Jaeschke et al. 2019; Käferböck et al. 2020; Akaberi et al. 2020). Moreover, Käferböck et al. conducted gate-to-gate life cycle assessment of production of 1 kg of extract and reported that PEF-assisted extracts had 57–65% lower environmental impacts compared to the unpretreated extracts due to the higher concentration of valuable compounds including proteins in the final extracts (Käferböck et al. 2020). The protein extraction from different microalgae was targeted by many authors including the PEF-assisted extraction (Coustets et al. 2013; Lam et al. 2017; Lam et al. 2017; Buchmann et al. 2019; Parniakov et al. 2015; Gateau et al. 2021; Coustets et al. 2015). The results of these studies have shown that the application of PEF to assist protein extraction was more effective compared to non-pretreated samples and less effective compared to mechanical, high hydrostatic pressure, and sonication-assisted extractions. However, Parniakov et al. (Parniakov et al. 2015) reported the improved selectivity of protein extraction from Nannochloropsis using PEF as pretreatment. Moreover, HVET can lead to the electrostimulation of microalgae, which can improve cell proliferation (Vorobiev and Lebovka 2020). Regarding macroalgae, several authors reported a significant improvement (2–4 times) of protein extraction when using PEF pretreatment coupled with mechanical pressing compared to untreated samples (Robin et al. 2018;

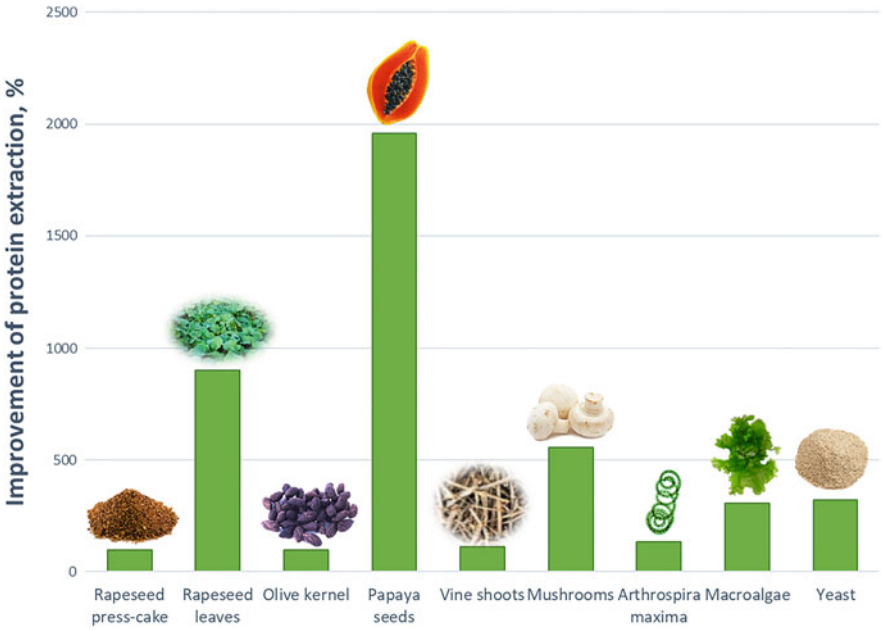

Fig. 8.3 Overview of improvement of protein extraction from various food matrices using HVET pretreatments (data combined from (Vorobiev and Lebovka 2020, Yu et al. 2015, Roselló-Soto et al. 2015, Parniakov et al. 2015, Xue and Farid 2015, Käferböck et al. 2020, Prabhu et al. 2019, Martínez et al. 2020))

Polikovsky et al. 2016; Prabhu et al. 2019). Moreover, as in the case of microalgae, the selectivity of protein extraction can be improved.

Protein extraction from bacteria and yeasts was demonstrated to be improved by PEF pretreatments. Martinez et al. (2020) have recently reviewed such approach. Indeed, the extraction of total proteins or certain enzymes from *E.coli* seems to be advantageous due to high protein yield, easier protein separation, no cell bacterial destruction (at certain PEF conditions) and lesser treatment duration compared to other pretreatment technologies. A similar tendency of increased protein release was reported on yeast strain *S. cerevisiae* pretreated with PEF. Moreover, as for the bacteria cells, the trade between protein yield and yeast cell viability should be found to optimize the process performance (Ohshima et al. 1995). Thus, yeasts found in multiple waste streams (e.g., brewer's, wine's and backer's yeasts) can be used as a source of valuable proteins, which can be efficiently extracted with the assistance of PEF. Indeed, several authors have reported the improvement of protein extraction (up to four times) from PEF pretreated yeasts compared to unpretreated yeasts (Martínez et al. 2016; Liu et al. 2012).

Thus, from above results, one can see that HVET lead to the improvement of protein extraction from protein-rich food matrices compared to unpretreated samples, which is resumed in Fig. 8.3. This fact means a substantial improvement of the value component of eco-efficiency. However, the utilization of HVET leads to

additional system and operational costs as well as environmental impact mostly associated with the energy consumption. Therefore, the final protein extracts should have a high value in order to be sustainably viable (especially from commercial and environmental points of view) to use HVET at industrial scale. Moreover, prior to such application the above-mentioned laboratory studies should be tested on an industrial scale equipment to find the optimal operational conditions allowing the maximization of protein yield. Additionally, the eco-efficiency of HVET should be compared with other emerging technologies, which allow the improvement of protein extraction. Furthermore, one should take into account the by-products generated after protein extraction. Indeed, often the protein-rich matrices contain various valuable compounds such as polyphenols and fibers demonstrating multiple biological activities (Vorobiev and Lebovka 2020; Barba et al. 2020). They could be as well extracted and used in the food, nutraceutical, or pharmaceutical applications. Otherwise, they can be used as animal feed or in biorefinery to improve the overall eco-efficiency of protein-rich matrices (Vorobiev and Lebovka 2020).

8.4 HVET Pretreatment of Proteins: Modulation of Functionality and Allergenicity

The more emergent application of HVET and especially PEF mode was reported to modify the functionality of protein-contained foods. The most explored direction of such protein modifications relates to the modulation of the activity of various enzymes (Table 8.1). Indeed, as reviewed by Zhao et al. (2012) and Zhang et al. (2021), the application of PEF at high electric field strength (up to 50 kV/cm) can lead to partial or complete inactivation of enzymes (e.g., lipoxygenase, peroxidase, polyphenol oxidase, papain) or, in some cases, an increase in enzymatic activity (e.g., horseradish peroxidase, lactate dehydrogenase). However, the mechanisms underlying such impacts are still not completely elucidated (Zhang et al. 2021; Zhao et al. 2012). The main causes of modulation of enzymatic activity are the modification of secondary and tertiary structures of enzymes due to the effects of applied potential differences such as polarization, redistribution of electron density and impact on different interactions occurring in protein molecules (e.g., dipole-dipole, electrostatic, hydrophobic) as well as the impact of species produced on the electrodes (H_2O_2, H^+, OH^-, O_3, etc.) (Giteru et al. 2018). Indeed, the loss of defined protein structures (e.g., α-helix and β-sheet) was reported by several authors, who studied the impact of HVET on enzymatic activity (Zhao et al. 2012). Castro et al. have proposed a model (Fig. 8.4) explaining the impact of PEF on the changes in protein molecules (Castro et al. 2001). In this model, the native protein (A) is subjected to an external high voltage electric field, which leads to polarization and changes of molecular dipole moment (B). Moreover, the application of a higher number of pulses leads to changes in the protein conformation and release of aliphatic amino acids from the protein core to the surface (C). Eventually, protein molecules can interact with each other via electrostatic and hydrophobic bonds and form aggregates (D, E). Thus, by varying the HVET parameters (e.g., electric field

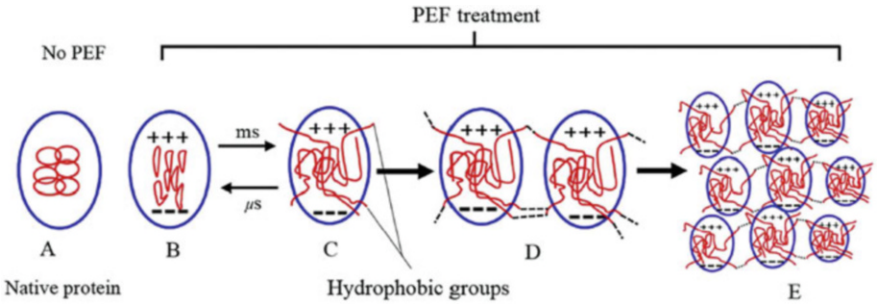

Fig. 8.4 Model of the impact of PEF on the protein molecules (Castro et al. 2001). Reprinted with permission from Giteru et al. (2018)

strength, pulse width, number of pulses, frequency, etc.), one can control to a certain extent the protein denaturation and the formation of aggregates (Giteru et al. 2018). It is worth noting that there is a possible significant temperature rise when applying HVET (especially PEF mode), which can act in synergy with the above-mentioned phenomena induced by an external electric field.

Another more innovative direction of HVET application to structural modifications of protein molecules concerns the impact on their functional properties. Indeed, the significant increase in solubility (1.12 times), hydrophobicity (1.25 times) as well as the concentration of surface content of sulfhydryl groups (1.63 times) was reported in soy protein isolates treated with PEF compared to untreated sample (Li et al. 2007). Zhang et al. reported the improvement of solubility (1.16 times), water-holding capacity (1.60 times), emulsifying capacity (1.13 times), emulsion stability (1.20 times), oil holding capacity (1.74 times), foaming capacity (1.40 times) and foam stability (1.51 times) of canola protein and its fractions after the pretreatment of canola seeds with PEF (Zhang et al. 2017). A recent study by Jin et al. (2020) reported that PEF can induce changes in the protein-polysaccharide interactions while studying the α-amylase-pectin complexes. The reduction of protein allergenicity by HVET treatments was recently targeted by several researchers. Indeed, Johnson et al. (2010) studied the impact of PEF on purified food allergens Ara h 2,6 (peanut 2S albumins), apple Mal d 1 and Mal d 3. However, no significant impact of electric field on the secondary structure of the studied allergens was detected. The structural changes of peanut proteins were studied by Vanga et al. (2015, 2016). The changes in the secondary structures of studied proteins were similar to or lesser than the ones that occurred during the thermal treatments. Pru p 3 protein from peach was also subjected to PEF alone and in combination with high temperature (50 °C) in order to reduce its allergenicity (Tobajas et al. 2020). Despite the structural changes induced by PEF at 50 °C, no impact on Pru p 3 protein allergenicity was observed. The above results suggest that more studies on the impact of PEF and arc treatments should be carried out in order to find the optimal conditions allowing the significant impact on the decrease in the allergenicity of plant proteins. Overall, the studies related to the functional properties and

allergenicity demonstrate promising application potential of HVET. However, the scaling up of this technology should be performed and eco-efficiency of obtained products should be evaluated and compared with products obtained via conventional and emerging technologies as discussed above for the HVET assisted extraction.

8.5 Impact of HVET on the Generation of Bioactive Peptides

HVET represent one of the most attractive alternatives to convert low-added value plant protein-rich products in valuable products containing peptides. Indeed, peptides are reported to possess multiple bioactivities promoting human health. For instance, peptides derived from plant proteins can possess antioxidant, anticancer, antihypertensive, opioid, antidiabetic, and other bioactivities (Piovesana et al. 2018). Moreover, consumer interest in such nutraceuticals is constantly raising, followed by the growth of the market for bioactive peptides, which is projected to surpass US$ 88.3 billion by 2027 (Coherent Market Insights 2020). Enzymatic hydrolysis of proteins remains one of the most abundant methods of peptide production. However, the main drawbacks standing in the way of valorization of protein-rich products via biocatalytic pathways are the high cost of enzymes and the very compact protein structure, which impedes the access of enzymes to their substrates (peptide bonds) in the protein molecules and prolongs the duration of hydrolysis. Therefore, HVET were very recently proposed as a method allowing to increase the susceptibility of proteins to enzymatic hydrolysis in order to improve the process performance (Mikhaylin et al. 2017). However, this application is still emergent for the plant proteins. So far, only Akaberi et al. studied the application of PEF to improve the enzymatic hydrolysis of proteins from algal biomass (Akaberi et al. 2019). These authors reported that PEF allowed a significant improvement of alcalase and flavourzyme hydrolyses of microalgae proteins. Other application of HVET to improve the enzymatic hydrolysis was recently demonstrated by Mikhaylin et al. and Agoua et al. on the β-lactoglobulin (main dairy whey protein) representing a family of globular proteins found in many plants. These authors have shown the substantial improvement (~ two times) of tryptic and chymotryptic hydrolyses of β-lactoglobulin when applying PEF and arc pretreatments compared to the control sample (Mikhaylin et al. 2017; Agoua et al. 2020). Moreover, the significant improvement of hydrolysis eco-efficiency of HVET pretreatments was reported compared to the conventional thermal treatments. However, applying a similar approach to proteins from different plants has yet to be explored.

The other emergent direction of HVET use is related to the improvement of the bioactivity of peptides released from different plant proteins (Zhang et al. 2021). Indeed, the application of high-frequency PEF allowed the substantial improvement (4–90%) of antioxidant activity of peptides derived from soybean and pine nut peptides. Moreover, the Alg-Gly-Ala-Val-Leu-His peptide from pine nut pretreated with PEF demonstrated the highest immunomodulatory activity compared to the non-pretreated one (Zhang et al. 2019). The positive impact of PEF treatments on peptide bioactivity can be explained by the structural changes in the peptide

molecules allowing the exposure of active groups of amino acids to the solution thus increasing the peptide bioactivity (Zhang et al. 2021). However, more detailed studies are necessary to deepen the mechanisms underlying the impact of HVET on the peptide bioactivities (e.g., molecular modeling, dipole moment and gyration radius measurements). Rivas et al. studied the impact of PEF on the stability of ACE inhibitory peptides in fortified complex beverage (Rivas et al. 2007). These authors reported that there was no impact of PEF on the ACE inhibitory activity of peptides present in the beverage. This fact suggests that in complex food solutions, the impact of PEF on the improvement of peptide bioactivity could be hampered and further investigations are needed to identify the causes.

As in the case of aforementioned applications of HVET technology, further research and scale up trials are needed to explore the full potential and eco-efficiency of such an innovative application dealing with high valuable peptides, which have multiple health-promoting effects.

8.6 Conclusion

The application of HVET for the valorization of plant protein-rich food matrices including large number of food by-products is a very promising way to improve the eco-efficiency of the obtained products due to the relatively low energy consumption of this technology, various possible applications at different stages of the protein processing (extraction, modification of functionality, production of bioactive peptides) allowing to substantially increase the value of the final product. Despite a large number of publications describing laboratory-scale studies concerning plant proteins, no industrial applications of HVET technology have yet been reported. Thus, further studies are needed to reveal the optimal processing conditions at larger scales as well as mechanisms of action of HVET, especially for emerging applications, including decreasing the protein allergenicity and proteolysis. Moreover, several studies concerning arc mode (or HVED) have presented very promising results compared to PEF mode, which is most probably due to additional phenomena occurring during arc (e.g., shock waves, Uv-light, cavitation bubbles). However, future research should focus on this special HVET mode to unlock its full potential. Additionally, some limitations of HVET (e.g., electrode corrosion, fouling, cost of equipment) should be taken into account before commercial applications of this technology (Pataro and Ferrari 2020). Eventually, the eco-efficiency assessment of plant proteins and peptides obtained using HVET technology is necessary to perform and compare with conventional and other emerging technologies to justify its utilization in the context of sustainable development.

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Microwave-Assisted Extraction of Plant Proteins

9

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Abstract

Due to the increase of world population, the demand for protein of animal origin is expected to double by 2050. However, the production of animal (meat, fish, and dairy) products has been associated to negative environmental impacts. This issue joined to new consumer trends towards healthy diets, has increased the search for plant protein sources as alternatives in food applications because of their high availability and low environmental impact. The efficient extraction of plant valuable proteins through suitable, cost-affordable, and sustainable technologies is desirable to comply with the progressively more stringent environmental, health, and safety regulations. This chapter aims to describe the recent developments on the extraction of plant proteins using an advanced green extraction technology such as microwaves, a cell disruptive technique that is proven to be more efficient with respect to protein recovery and producing minimal environmental impact. This chapter will overview the basic principles of microwave extraction, operating parameters, types of equipment that are available, advantages and limitations when compared to other traditional protein extraction techniques. Moreover, the impact of the technology on the protein recovery for different sources (cereals, oilseeds, pulses, algae, agro-industry by-products) will be summarized. Finally, the level of readiness for industrial application and research avenues will be proposed.

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Keywords

Microwave-assisted extraction · Protein · Plant raw materials · Agri-food by-products

Abbreviations

2-HEAA	2-hydroxyethylammonium acetate
2-HEAF	2-hydroxyethylammonium formate
ATP	aqueous two-phase systems
EAA	essential amino acids
HPMAE	high-pressure microwave-assisted extraction
IL	ionic liquids
MAE	microwave-assisted extraction
NEAA	non-essential amino acids
PEG	polyethylene glycol
RSM	response surface methodology
TPP	three phase partitioning
UMAE	ultrasound microwave-assisted extraction.

9.1 Introduction

The first evidence on microwave heating was reported in the 1940s, although applications of microwaves in extraction processes of target organic compounds from several matrices initiated in the late 1980s. Microwave is a radiation in which electric and magnetic fields oscillate perpendicularly in a range of frequencies from 0.3 to 300 GHz. Microwave frequencies have specific regulation to avoid interference between different radio waves. In this context, 2450 MHz and 915/2450 MHz are employed usually for home cooking and industry, respectively (Guo et al. 2017; Pu et al. 2016).

Microwave energy has effects on molecules known as dipolar rotation and ionic conduction (Leonelli et al. 2013). The electromagnetic field is responsible for the rearrangement of dipoles (dipolar rotation) and the migration of charged colloidal molecules through a stationary medium (ionic conduction). These effects produce a flow of charged molecules that causes resistance in the solution and the subsequent transformation of microwave energy into thermal energy. Generally, the loss tangent (tg δ) of the material defined as the ratio of the dielectric loss (ε'') and dielectric constant (ε') will determine its heating properties under microwave conditions. Dielectric constant is associated to the ability of the material to store electric energy, whereas dielectric loss factor measures the capacity of the material to dissipate electric energy. Based on their high dielectric constant, polar materials (salts, water, alcohols, etc.) are microwave active, whereas non-polar materials with poor

dielectric constant are microwave transparent and cannot be heated using this technology (Leonelli et al. 2013). Polar or charged molecules in materials rotate back and forth under microwave, causing the friction and collision between them which is transformed quickly in heat transfer to the material (Bhattacharya et al. 2017). Moreover, the rapid penetration of microwaves makes it more efficient than the conventional heating (Tang 2015). Consequently, microwave heating has higher efficiency allowing saving energy not only during cooking of foods but also in other applications such as the extraction of organic compounds.

Microwave-assisted extraction (MAE) based on the combination of dielectric heating and solvent extraction of target compounds from various matrices has been used for the last decades. It has shown great advantages compared to conventional extraction, such as enhanced extraction yields, shorter extraction time, and reduction of energy consumption and solvent usage (Li et al. 2019). The increased efficiency of MAE as compared to conventional extraction methods is the result of the synergic action of heat and mass transfer that occur in the same direction. Furthermore, heat is dissipated volumetrically inside the solid matrix (Veggi et al. 2013). During MAE, a series of steps take place (Fig. 9.1), including: (1) the solvent penetration into the solid matrix; (2) the breakdown of solutes interaction with sample matrix (3) the solubilization of solutes from the sample matrix to the solvent; (4) the internal diffusion of the extract across the sample matrix; (5) the external diffusion of the extract from the matrix to the solid; (6) the separation and discharge of the extract and solid.

In plant matrices exposed to microwave irradiation, dipole rotation breaks down hydrogen bonding, whereas ion migration favors the solvent penetration into the matrix and the solubilization of intracellular material into the solvent media. Since plant matrices contain water (polar solvent), microwave energy is absorbed quickly producing an internal superheating that continues with a rapid evaporation, high intracellular pressure, and cellular disruption in the plant matrix (Chew et al. 2019; Chan et al. 2016). Chan et al. (2016) developed a theoretical model to describe the mechanism of cell disruption during MAE of phytochemicals from plant matrices. From this model, it was demonstrated that the energy needed for cellular disruption

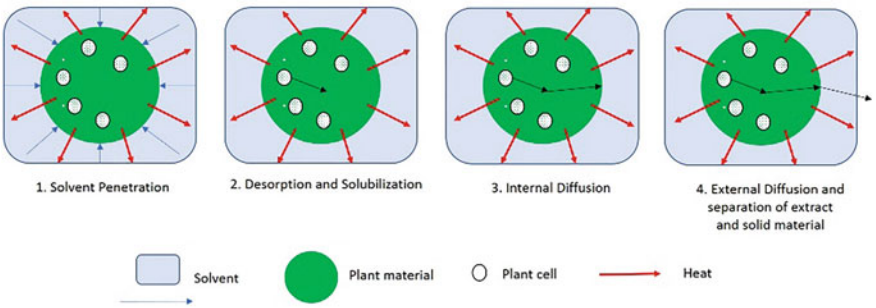

Fig. 9.1 Basic mechanisms of heat and mass transfer in microwave-assisted extraction of natural compounds

is mainly dependent on the mechanical properties of plant cell walls. Thus, small plant cells with a thick cell wall will require more energy to be disrupted. Additionally, the model was calibrated for an accurate prediction of the optimum extraction time and could be applied over a wide number of operating parameters, regardless of the type of microwave system. Cellular disruption contributes to MAE efficiency as not only increases cell porosity that facilitates the accessibility of solvent into the cells but also enhances contact area between solid and liquid phase, the release of intracellular compounds in the solvent system, and the internal and external diffusion of metabolites (Chew et al. 2019; Chan et al. 2016).

Several excellent books and reviews have been published over the past 10 years describing the contemporary developments in MAE of aromas, essential oils, alkaloids, fat and oils, antioxidants, pigments, and other bioactive compounds (Veggi et al. 2013; Li et al. 2019). More recently, MAE has been recognized as a promising alternative to conventional extraction methods for isolation and purification of protein from cereals, legumes and oilseeds crops, novel plant biomasses (green leaves, microalgae, seaweeds) and agri-food by-products and waste (Chew et al. 2019).

This chapter will overview the basic principles of microwave extraction, operating parameters, type of equipment that are available, advantages and limitations when compared to conventional protein extraction techniques. Moreover, the impact of the technology on the protein recovery for different plant sources (cereals, oilseeds, pulses, algae, agro-industry by-products) will be summarized. Finally, the level of readiness for industrial application and research avenues will be proposed.

9.2 Operating Parameters

MAE efficiency relies on the selection of operating conditions and parameters that maximize not only protein yield but also final product quality and safety. The factors that may influence MAE efficiency are solvent system, solvent to feed ratio, microwave power and temperature, extraction time and cycle and plant matrix (particle size and moisture content). Comprehension of the effect of these parameters in the MAE process and its relationship with nutritional, physicochemical, and safety attributes of final products is important for an efficient production of high-quality protein ingredients from plant matrices. Several studies have applied statistical methods, such as response surface methodology (RSM), to identify the optimal operating conditions for maximal extraction yields of proteins. Optimal operating conditions for MAE of proteins reported in the literature so far are collected in Tables 9.2, 9.3, and 9.4 and described in Sect. 9.4.

9.2.1 Solvent System and Solvent to Feed Ratio

Solvent system is the main factor that influences the efficiency of MAE. Selection of solvents should be made based on a high dielectric constant, dielectric loss and selectivity towards the stability and solubility of extracted proteins (Chen et al. 2008). Aqueous solutions are frequently used for plant protein extraction, whereas organic solvents (ethanol, butanol, and acetone) are selected to extract proteins having aromatic, non-polar, and polar amino acid residues (Cui et al. 2017). Alkali (NaOH or KOH) are commonly used solvents for plant protein extraction due to their higher yield (Kumar et al. 2021a). Furthermore, salts such as NaCl can be added to improve the protein recovery (Fetzer et al. 2018). Basic pH produces the breakdown of disulfide bonds in proteins and the increase in protein solubility due to ionization of acidic and neutral amino acids (Contreras et al. 2019). High extraction yields (>90%) of proteins from cereal, legume and oilseed crops and their by-products or residues (leaves, bran, etc.) have been reported in alkaline conditions. Nevertheless, their use may negatively affect protein quality, functionality, and consumer acceptability. In particular, alkaline protein extraction produces protein denaturation, cross-linking, hydrolysis of peptide bonds, and loss of amino acids (Contreras et al. 2019).

Three phase partitioning (TPP) has gained considerable attention as a simple and quick technique for small and large-scale separation and purification of proteins (Chew et al. 2019). This solvent system uses equal volumes of large concentrations of well-buffered aqueous and alcoholic solutions. TPP is formed when the two liquid solutions are separated by an intermediate layer of precipitated protein (Dennison and Lovrien 1997). In TPP systems, *t*-butanol is usually the component of the alcoholic solution due to advantages such as its branched structure that impedes the permeation of alcohol molecules into the folded proteins resulting in high product quality (Chew et al. 2019). The composition of the aqueous solution (type of salt and concentration of alcohol and salt) in TPP systems has been shown to be a critical factor for MAE affecting protein recovery yield and separation efficiency. In particular, a concentration of 30% ammonium sulfate in the TPP systems resulted in higher extraction yield and efficiency when proteins from *Chlorella vulgaris* microalgae were extracted by microwave technology (Chew et al. 2019). These results were attributed to the good hydration of the sulfate ions that produce salting out effects in the proteins, a phenomenon that reduces free water available to dissolve proteins flocculating and separating them to the intermediate phase. Salting out effects increased with salt concentration leading to high recovery yield, although too high salt concentrations could produce irreversible denaturation of proteins by excessive dehydrating effect and sulfate ions pushing them to the alcohol phase (Chew et al. 2019). *t*-Butanol ratio also influences protein extraction yield of microwaves in TPP when microalgae biomass was used as the protein source (Chew et al. 2019). A solvent ratio of 1:1 produces better outcomes, whereas higher *t*-butanol concentration could result in protein denaturation, decreased absorption of microwave energy in the microalgae biomass, lower heat transfer and cellular disruption.

Ionic liquids (IL) are being increasingly used due to their unique chemical and physical properties, such as tunable viscosity, good thermal stability, negligible volatility, and preservation of biological activities (Freire et al. 2012; Passos et al. 2014). IL have also received attention in a limited number of applications in MAE of phycobiliproteins from microalgae biomass (Motlagh et al. 2021; Martins et al. 2016), reducing not only environmental impact, but also improving extraction efficiency. Different families of IL (piperidinium, quaternary ammonium, imidazolium, pyridinium, pyrrolidinium, phosphonium, and cholinium) have been used to recover *Gracilaria sp.* phycobiliproteins (Martins et al. 2016). Cholinium chloride was the most promising IL to increase extraction efficiency up to a maximum of 46.5% while maintaining the chromophore conformation integrity and protein secondary structure.

The solvent-to-solid ratio is also a key parameter to be considered in MAE optimization. The increase in solvent volume improves the extraction recovery yield, although a large volume of the extracting solvent could give lower recoveries because of non-uniform distribution and exposure of microwaves (Eskilsson et al. 1999). In many applications, a ratio 10:1 or 20:1 (mL:g) was found to be optimal (Kumar et al. 2021a). In other cases, smaller amounts of solvent (3:1, mL:g) are sufficient to extract the proteins of interest (Mahali and Sibi 2019).

9.2.2 Extraction Time and Cycle

Extraction time is a critical factor in MAE of organic compounds. Optimal time for protein extraction from plant matrices varies from 10 seconds to 30 min (Tables 9.2, 9.3, and 9.4). Excessive long microwave time could lead to excessive heating and denaturation of proteins and release of impurities, causing a reduction in protein yield (Patil and Yadav 2018). In contrast, insufficient microwave irradiation may prevent cell disruption, making the release of proteins ineffective. Occasionally, when longer extraction time is required, the constant molecular friction increases the temperature and degrades thermal sensitive proteins. Hence, when using consecutive extraction cycles reduces the energy consumption and evaporation of solvents (Chan et al. 2011). With this procedure, the extraction yield might be improved, avoiding long heating. A related work of Chew et al. (Chew et al. 2019) investigated the effect of microwave duty cycle at 20, 40, 60, 80, and 100% duty cycle for a microwave time of 120 s and power of 180 W on extraction yield of proteins from *Chlorella vulgaris*. A lower protein yield was observed for duty cycles from 20% to 60% (18 s ON; 12 s OFF), where 20% duty cycle had the lowest yield of 36.3%. For 80% duty cycle (24 s ON, 6 s OFF), the yield increased slightly (51.9%) compared to the full cycle of microwave irradiation. Slightly lower yield for 100% duty cycle compared to 80% duty cycle could be due to the prolonged microwave dose, in which using the pulse mode can effective provide a temporary stop in the irradiation to avoid solvents evaporation and reduce the molecular friction, which may disrupt the protein content in the solution.

9.2.3 Microwave Power and Extraction Temperature

Typically, microwave power applied for protein extraction varies from nearly 100 W to 1000 W (Tables 9.2, 9.3, and 9.4). Microwave power is positively associated to extraction temperature and both are usually optimized to maximize the protein yield, energy, and operational costs. Increases in temperature facilitate solubilization of solutes due to a drop in solvent surface tension and viscosity, which favors its diffusion in the food matrix (Li et al. 2010). Solvents may reach the boiling point when MAE is carried out in closed vessels, improving extraction efficiency due to desorption of compounds from the plant matrix (Sparr Eskilsson and Björklund 2000). Generally, efficiency increases with increasing temperature levels up to an optimum value where it begins to decrease (Routray and Orsat 2012). This behavior is directly related with protein thermal stability and, therefore, with the recovery yield. Apart from thermal denaturation, microwave irradiation can also affect the structure of proteins and peptides, leading to protein misfolding, association, and aggregation. On the other hand, higher microwave power could produce a more rapid increase in temperature and rupture of the cell wall, favoring the co-extraction of impurities to the solvent (Veggi et al. 2013).

Microwave power is also directly related to the amount of feedstock and the extraction time required. Excessive feedstock mass will require higher microwave power for cell disruption to improve the extraction yield and reduce extraction time (Bhattacharya et al. 2017; Baiano 2014).

9.2.4 Particle Size and Water Content of the Plant Matrix

The intrinsic features of the plant matrix influence MAE process efficiency and protein recovery yield. Plant matrices with a smaller particle size and higher contact surface area allow a deeper penetration of the microwaves and solvent, resulting in an increase in the extraction efficiency (Huie 2002). Recommended particle sizes are between 0.1 and 2 mm (Chan et al. 2011). Therefore, plant matrices are usually grinded and homogenized to maximize the contact area with the solvent. After grinding, dry plant materials are often soaked in water or alcoholic solutions before MAE as the increase in moisture content of the plant matrix usually results in an improved extraction yield (Chan et al. 2011).

9.3 Microwave Equipment and Systems

MAE equipment (Fig. 9.2) is constituted of four main components: a generator of microwaves; a waveguide needed for microwave propagation from the source to the cavity; a sample incubator; and a circulator that allows the microwaves to move only in the forward direction.

MAE is categorized in multi-mode and mono-mode systems. In the former, microwave irradiation is dispersed randomly in cavity by a mode stirrer that operates

Fig. 9.2 Continuous microwave-assisted extraction equipment (Reproduced with permission of Sairem, Decines—Charpie, France; website: https://www.sairem.com/industrial-scale-continuous-microwave-assisted-plants-extraction/)

at high pressure (closed system), whereas in the latter system, irradiation is focused in a restricted area in cavity and usually operates at atmospheric pressure (open system).

In the closed system, treatments are performed at controlled high-pressure and temperature conditions in closed vessels which offer less solvent consumption and fast and efficient extraction. Recent technological developments of the closed system are known as high-pressure microwave-assisted extraction (HPMAE).

Open systems operate at mild conditions being more suitable to extract thermolabile compounds. Moreover, they have larger sample throughput and allow the continuous addition of solvent during the process (Chan et al. 2011).

Several modified microwave extraction systems have been developed to improve protein extraction yield (Baiano 2014). An example is ultrasonic MAE (UMAE) that combines microwave and ultrasonic waves to promote cell rupture and enhance the interaction of solvent and intracellular material in a shorter time. This technique has been employed in the protein extraction from *Moringa oleifera* leaves (Dai et al. 2020) and *Chlorella vulgaris* biomass (Stramarkou et al. 2017). For UMAE of proteins, processing parameters such as extraction time have the largest influence on protein yield, followed by microwave power, solvent-solid ratio, and temperature. Longer irradiation time and excessive temperature for UMAE should be prevented as they will accelerate protein denaturation and precipitation (Lv et al. 2019).

Upscaling of MAE system to industrial production is feasible and provides economic and environmental benefits based on the lower cost for manufacturing high-quality bioproducts and lower energy usage (Li et al. 2013). To achieve the

desired benefits and outputs, implementation of MAE technology in industrial production needs to be studied individually for each system (Radoiu et al. 2019). Several studies have demonstrated that MAE of polyphenols (Périno et al. 2016), essential fatty acids (Filly et al. 2014), volatile and non-volatile organic compounds (Petigny et al. 2014), pectin and limonene (Ciriminna et al. 2016) is feasible at pilot and industrial scales. However, scalability of MAE for commercial production of plant protein isolates/concentrates has been less explored (Li et al. 2012). Up to date, there have been advances in the development of MAE systems that solve the low penetration of microwaves operating at a frequency of 915 MHz. In addition, new designs of microwave generators with an output power up to 120 kW/unit has allowed large-processing capacity (up to 870 kg/h) (Radoiu et al. 2019). Other innovations performed in microwave reactors is the integration of other technologies such as vacuum, ultrasounds, or supercritical conditions that improve extraction efficiency (Bagade and Patil 2019). Designs of MAE systems should be specifically performed to assure controllability and monitoring of the process conditions which require considerations on temperature of heated mixture, power dissipation rate, and electric field intensity because they impact microwave reactor output and its scale (Chan et al. 2011).

9.4 Microwave-Assisted Extraction Vs. Conventional Extraction of Proteins

Nowadays, food industry produces three types of protein ingredients including protein concentrates, isolates, and hydrolyzates through three main steps: protein extraction, separation, and drying. The disruption of cellular structure and dissociation of proteins from lipid, polysaccharides, and pigments in an efficient and sustainable way present major challenges (Tamayo Tenorio et al. 2018). Large amounts of insoluble polysaccharides are a challenge in plant protein processing, due to the viscosity of these macromolecules or their interactions with proteins. On the other hand, raw materials from plant origin are a source of natural phenolic compounds that also interact with hydrophobic groups of proteins, changing their physicochemical properties, including structure, solubility, thermal stability, and digestibility. Therefore, the extraction technique used influences the protein yield and product quality. The physical properties of the solvent (surface tension, viscosity, and vapor pressure) and temperature of extraction directly affects extraction efficiency and physicochemical, rheological, functional, and structural properties of proteins.

Alkaline and saline solutions are employed in conventional protein extraction, whereas ultrafiltration/diafiltration techniques and isoelectric and micellar precipitation are the most common methods used for protein separation. Protein recovery and final product quality depend on the cellular matrix and the applied technology. Major drawbacks/limitations of conventional extraction techniques include low extraction yield and selectivity, long extraction periods, and thermal degradation of thermolabile compounds (Guo et al. 2017). From an environmental perspective, conventional

Table 9.1 Advantages and disadvantages of conventional solvent extraction vs. MAE

	Advantages	Disadvantages
Conventional solvent extraction	• Inexpensive and simplicity. • Allows for solvent reuse.	• Does not always use generally recognized as safe solvents. • Frequently requires an evaporation/concentration step for recovery. • Usually demands large amounts of solvent and long extraction time. • Possibility of thermal degradation.
MAE	• Reduced extraction time (75–80%). • Reduced solvent usage. • Selective heating. • Reduced floor space of the installation. • Improved extraction yield. • Lower production cost due to waste decrease and production losses. • Reduction of the footprint (50–90%). • Saving of electric energy (25–50%).	

Source: Zuin and Ramin (2018), Li et al., (2012)

protein extraction techniques have unavoidable drawbacks including its time-consuming, energy-consuming, and high solvent consumption features.

To address drawbacks of conventional techniques, MAE has been proposed in the recent literature. This extraction technique is considered "green" in nature (Behere et al. 2021), as comply with standards set by the US Environmental Protection Agency.

Compared to conventional extraction techniques, the major advantages of MAE include overall cost-effective ratio and eco-friendly processing conditions (reduced extraction time and solvent volume, use of safer solvents, energy efficiency, higher efficacy, prevention of thermal degradation, etc.) (Table 9.1).

9.5 Impact of Microwaves Technology on Different Plant Sources

9.5.1 Cereals, Pulses, and Other Plant Sources

Cereals are one of the resources for human nutrition all around the world owing to their excellent nutritional profile, being proteins one of the most outstanding nutrients in their composition. The content of proteins in cereals varies between 6 and 15% depending of the cereal considered (Shewry 2007), thus they constitute

suitable food sources for obtaining proteins concentrates or isolates. Cereal proteins are often aggregated or linked to other compounds such as cell wall polysaccharides or starch granules that limit their solubility (Branlard and Bancel 2007). Therefore, the application of technological methods to improve protein solubility is of great interest for recovery of proteins from cereal grains. Microwaves have been shown to effectively assist protein extraction from different plant-based sources (Bedin et al. 2020; Bandyopadhyay et al. 2012; Wen et al. 2020) due to the disruption of cell membrane, reducing the time required for leaching proteins into solvent (Behere et al. 2021). However, MAE has been hardly explored to improve cereal protein extractability so far. Lamacchia et al. (2016) observed that microwave treatment applied to hydrated wheat kernels broke hydrogen bonds between protein glutamine residues present in their native form in protein bodies, thus favoring their solubilization in aqueous saline solutions (Table 9.2).

Only a few studies have investigated the application of MAE for improving the recovery of proteins from pulses (Table 9.2). An improvement of soluble soybean protein recovery was observed after application of MAE to six different soybean cultivars as compared to conventional extraction by shaking in a water bath (Choi et al. 2006). Protein yield increased as temperature, time, and sample/solvent ratio augmented until 60 °C, 30 min and 12 mg soybean flour/mL, respectively, and then decreased with further increases of temperature and sample/solvent ratio due to protein denaturation. Disruption of cell wall and plasma membrane and the release of storage proteins from protein bodies could be responsible for the enhancement of protein recovery by MAE. Similarly, a significant increase in protein extraction yield (4.8 kg/kg soybean in MAE vs. 3.7 kg/kg soybean in conventional extraction) and protein content (13.1% in MAE vs. 7.4 in conventional extraction) was noticed in milled soybean treated by microwave heating during soybean milk elaboration in comparison with conventional extraction method (Varghese and Pare 2019). The optimal MAE conditions identified by the authors to achieve the maximal protein recovery were power level of 675 W, temperature of 80 °C, and stirring speed of 160 rpm. A recent study demonstrated that application of microwaves at 900 W for 3 min on soybean kernels followed by thermosonication at 60 °C for 30 min effectively improved protein solubility and other physicochemical properties in soymilk, reducing at the same time trypsin inhibitors and lipoxygenase activity (Kumar et al. 2021b). MAE also resulted in higher protein extraction yield from peanut flour as compared with control process (alkaline extraction) (Ochoa-Rivas et al. 2017). In particular, MAE application at 725 W for 8 min extracted 77% more protein than the conventional extraction process. In contrast, Sun et al. (Sun et al. 2020) did not observe any improvement of protein extraction yield after treatment of pigeon pea (*Cajanus cajan*) flour by microwaves for 3 min as compared to control untreated flour. However, this treatment improved protein digestibility from 54% to 72% due to the particle size reduction and secondary structure changes induced by microwave heating.

Recent studies have recognized MAE as an optimum choice for the protein extraction from herbal plants. Through a Circumscribed Central Composite design, Elhag et al. (Elhag et al. 2019) developed an MAE methodology to extract protein

Table 9.2 Application of MAE for recovery of proteins from cereals, pulses, and other plant-based sources

Feedstock	Solvent used	Ratio (solid/solvent)	MAE optimal conditions	Main results (PC/PR/PY)	Ref.
Wheat kernel	Soaked kernels (18–20% humidity)	–	1000 W, 110–120 °C, 2 min	Increased protein solubilization in aqueous saline solutions	(Lamacchia et al. 2016)
Soy (6 cultivars)	Distilled water	12 mg/mL	2450 MHz, 60 °C, 30 min	PC: 28–40 mg/mL	(Choi et al. 2006)
Soymilk	Soaked grains	–	2450 MHz, 675 W, 60 °C, 35 min	PY: 4.83 (kg/kg) PC: 13.12%	(Varghese and Pare 2019)
Soymilk	Distilled water	20 g/50 mL	MAE (900 W, 3 min) and US (28 kHz, 60 °C, 90 min)	Enhancement of physicochemical properties Reduction of TI and LOX activities	(Kumar et al. 2021b)
Peanut flour	Distilled water	1:10–1:25 w/v	725 W, 8 min	PY: 55%	(Ochoa-Rivas et al. 2017)
Pigeon pea	Deionized water	20%, w/v	Cook mode, 3 min	Decrease (49%) of protein solubility Increase (32% of protein digestibility)	(Sun et al. 2020)
Jackiopsis ornata roots	Distilled water	1:30, w/v	300 W, 65 °C, 20 min	PY: 20.43%	(Elhag et al. 2019)
Eurycoma apiculate roots	Deionized water	1:20, w/v	270 W, 46 °C, 19 min	PY: 15.85%	(Abugabr Elhag et al. 2020)
Moringa oleifera leaves	0.15 M Tris-HCl	1:128 w/v	81 W, 41 °C, 148 s	PY: 82.07 mg/g	(Cheng et al. 2021)

LOX lipoxygenase, *PC* protein concentration, *PY* protein yield, amount of protein extracted per mass of feedstock, *TI* trypsin inhibitor, *US* ultrasonication

from *Jackiopsis ornate* roots. These authors obtained the highest protein yield (20%) with a solid-to-liquid ratio of 1:30 (w/v), temperature of 65 °C, microwave power of 300 W, and extraction time of 20 min. A subsequent study of these authors illustrated the high efficiency of MAE to recover the water-soluble proteins from the root extracts of the medicinal plant *Eurycoma apiculata* with low solvent volumes and short extraction time (Abugabr Elhag et al. 2020). The highest yield (16%) was found when MAE was applied for 19 min at 61 °C, and under a power

level of 430 W. *Moringa oleifera* leaves have been reported to contain significant amount of high-quality protein useful as supplements and food functional ingredients, thus optimization of its extraction is of great interest. Very recently, Chen et al. (Cheng et al. 2021) compared the protein yields resulting from conventional solvent and UMAE from leaves, finding that the novel methodology exhibited significantly high extraction efficiency (82 mg/g vs. 69 mg/g) with low energy and time costs.

9.5.2 Algae

Algae are a group of photosynthetic organisms that are preferentially aquatic, although they can colonize other environments including highly acidic and frozen soils (Levasseur et al. 2020). They are distinguished into macroalgae, commonly referred to as seaweed, which are macroscopic and multicellular organisms, and microalgae, which are microscopic and unicellular organisms. Seaweed and microalgae are attractive sources of high-quality protein alternative to traditional protein sources since they can accumulate protein levels similar to meat, eggs, and milk (Bleakley and Hayes 2017). Algae exhibit some advantages over other crops for protein extraction since they can be cultivated on non-arable land without fresh water, show high growth rate and productivity (Chia et al. 2019), and reduce the emission of greenhouse gas (carbon dioxide) in atmosphere (Daneshvar et al. 2022). For these reasons, they have interesting commercial applications in nutraceuticals and functional foods.

Protein content of microalgae varies depending on the species considered, but it can reach 43–71% in *Chlorella* sp. and *Spirulina* sp. (Chia et al. 2019; Daneshvar et al. 2022; Christaki et al. 2011). In the case of seaweeds, protein content varies from 3–21% in brown seaweeds, 4–44% in green seaweeds, and 8–47% in red seaweeds (Tamayo Tenorio et al. 2018). Microalgae and seaweed proteins contain essential amino acids (EAA), including threonine, methionine, isoleucine, valine, leucine, lysine, and histidine (Daneshvar et al. 2022; Christaki et al. 2011; Safi et al. 2013; Kadam et al. 2013) meeting Food Agricultural Organization composition requirements (FAO/WHO 1991). They also contain non-essential amino acids (NEAA) such as arginine, proline, glutamic and aspartic acids, cysteine, and glycine (Safi et al. 2013). Even though their potential, microalgae have been underexploited as a protein source since they are intracellular compounds, and their extraction requires the disruption of rigid cell walls that are often resistant to chemicals and weak acids/bases (Kapoore et al. 2018), and this step involves a large proportion of the total processing costs. Conventional techniques used for protein recovery from microalgae such as membrane separation, column chromatography, precipitation, crystallization and ultrafiltration assisted by mechanical, physical, or enzymatic treatments to lyse cell wall require multiple processing steps and, consequently long processing times and protein loss throughout the process, as well as the utilization of hazardous organic solvents, and are difficult to scale-up (Chia et al. 2019; Chew et al. 2019).

Table 9.3 summarizes the main studies performed in last years focused on protein extraction from different microalgae by using MAE. Several microalgae are valuable sources of phycobiliproteins that are commonly used as food natural pigments, pigments for cosmetics, fluorescent reagents, and also as nutraceuticals due to their health-promoting properties (Spolaore et al. 2006). Recently, MAE has been used for obtaining phycoerythrin, phycocyanin, and allophycocyanin from *Porphyridium purpureum* (Juin et al. 2015). High extraction yield of phycoerytrin (73.7 μg/mg) was observed after application of MAE at 40 °C for 10 seconds on *Porphyridium purpureum* freeze-dried cells, while the maximal extraction yield for phycocyanin (34.8 μg/mg) and allophycocyanin (35.1 μg/mg) was found after MAE at 100 °C for 10 seconds and 1 min, respectively. These authors demonstrated that the use of MAE accelerated phycobiliproteins extraction 1.1-fold, and increased extraction yield between 6 and 29-fold compared with the traditional soaking extraction method. Esquivel-Hernández et al. (Esquivel-Hernández et al. 2017) also optimized MAE operating conditions using polar (ammonium acetate 10 mM and ethanol, 0.25 and 0.81, v/v) and non-polar (limonene and ethyl acetate, 0.25 and 0.81, v/v) solvents for maximizing the recovery of phycocyanins from *Arthospira platensis*. The results revealed that the optimal MAE conditions were 400 W, 1 bar, 15 min at 60 °C and 40 °C for the highest extraction of C-phycocyanin (2.3 μg/g) and A-phycocyanin (4.1 μg/g), respectively. Similarly, a novel MAE method using protic IL as solvent has been proposed for recovery of phycobiliproteins from *Arthrospira platensis* (Rodrigues et al. 2020). The efficacy of 3 different protic IL: 2-hydroxyethylammonium acetate (2-HEAA), 2-hydroxyethylammonium formate (2-HEAF, their equimolar mixture (2-HEAA +2-HEAF), and the commercial IL 1-butyl-3-methylimidazolium chloride was examined in this research. The process conducted at 62 W and pH 7.0 using the mixture 2-HEAA +2-HEAF as solvent and a solvent to biomass ratio of 10 mL/g conducted to the largest phycobiliprotein extraction (0.84 g/L, 1.33 g/L and 0.41 g/L for phycocyanin, allophycocyanin and phycoerytrin, respectively). Mahali and Sibi (Mahali and Sibi 2019) obtained high protein yield (>75%) from *A. platensis* after application of MAE at 1000 W for 3 min. MAE (900 W, 3 min) has also been effectively used for extraction of soluble proteins from a mixed culture of green microalgae *(Stigeoclonium* sp. and *Monoraphidium* sp.) and diatoms *(Nitzxchia* sp. and *Navicula* sp.*),* being the protein yield 18-fold higher than in control extraction (without microwave application) (Passos et al. 2015).

In another study, the efficiency of MAE along with TPP was evaluated for the extraction and purification of proteins from *Chlorella vulgaris* FSP-E microalgae strain (Chew et al. 2019). MAE-TPP system used in this study comprises three phases consisting of a salt phase, where five different salts were studied (ammonium sulphate, sodium sulphate, magnesium sulphate, magnesium acetate, and dipotassium hydrogen phosphate), and a t-butanol phase. The highest protein recovery was achieved by using ammonium sulphate concentration of 30% w/v, ratio of slurry (microalgae in ammonium sulphate) to t-butanol of 1:1, microwave irradiation time of 120 s, microwave duty cycle of 80%, microwave power of 100 W, and initial microalgae biomass concentration of 0.5 w/w. Under these conditions, the protein

Table 9.3 Application of microwave-assisted extraction for recovery of proteins from different microalgae

Microalgae	Solvent used	Ratio (solid/solvent)	MAE optimal conditions	PC/PR/PY	Ref.
Arthrospira platensis	Ammonium acetate 10 mM + ethanol (0.25 v/v)	0.14 w/v	400 W, 1 bar, 60 °C, 15 min 400 W, 1 bar, 40 °C, 15 min	PC (C-phycocyanin): 2.28 μg/g PC (A-phycocyanin): 4.11 μg/g	(Esquivel-Hernández et al. 2017)
Arthrospira platensis	Distilled water	5 g dm/15 mL	1000 W, 3 min	PY: >75%	(Mahali and Sibi 2019)
Arthrospira platensis	2-HEAA +2-HEAF (1:1 v/v)	1 g/10 mL	62 W, pH 7.0	Allophycocyanin PC: 1.33 g/L; PY: 13.30 mg/g Phycocyanin PC: 0.84 g/L; PY: 8.40 mg/g Phycoerythrin: PC: 0.41 g/L; PY: 4.10 mg/g	(Rodrigues et al. 2020)
Chlorella vulgaris FSP-E	Ammonium sulphate + t-butanol	1:1 v/v	100 W, 120 s,	PY: 63.2%	(Chew et al. 2019)
Nannochloropsis oceanic	2% choline acetate	0.5 g/15 mL	700 W, 2450 MHz, 40 °C, 30 min	PY: 26.35% PR: 65.06%	(Motlagh et al. 2021)
Porphyridium purpureum	Deionized water	20 mg/7 mL	40 °C, 10 s	PY (phycoerythrin): 73.7 μg/mg dm	(Juin et al. 2015)
		20 mg/7 mL	100 °C, 10 s	PY (phycocyanin): 34.8 μg/mg	
		20 mg/7 mL	100 °C, 1 min	PY (allophycocyanin): 35.1 μg/mg	
Stigeoclonium sp., *Monoraphidium* sp., *Nitzxchia* sp., and *Navicula* sp. mixed culture	Wastewater	–	900 W, 3 min (34.3 MJ/kg TS)	PC: 193 mg/L	(Passos et al. 2015)

2-HEAA 2-hydroxyethylammonium acetate, *2-HEAF* 2-hydroxyethylammonium formate, *dm* dry matter, *PC* protein concentration, *PR* protein recovery, amount of protein extracted per total amount of proteins, *PY* protein yield, amount of protein extracted per mass of feedstock, *TS* total solids

recovery yield from *Chlorella vulgaris* FSP-E was found to be 63.2%, which was 2.5-fold higher than in TPP process. More recently, IL-based MAE was carried out under different conditions of temperature (30–80 °C), extraction time (5–35 min) and IL concentration (0–4.7% w/v) as a promising protein extraction method from *Nannochloropsis oceanica* (Motlagh et al. 2021). Among the six types of IL evaluated, choline acetate was the most effective for protein extraction and the highest protein yield (26.4%, representing around 65% of the total *N. oceanica* proteins) was obtained with 2% of choline acetate, 0.5 g of microalgae and microwave irradiation at 700 W and 40 °C for 30 min. The protein yield obtained with this innovative extraction method was superior to that obtained with a conventional hexane extraction method (0.6%).

9.5.3 Agro-Industry by-Products

The agri-food industry generates annually a huge amount of by-products (peals, pomace, leaves, kernels, seeds, skins, bones, and other inedible fractions) that are discarded, causing a global environmental concern and a socioeconomic negative impact (Gullón et al. 2020). With the concept of sustainable development and circular economy, novel approaches towards their valorization into high-value products are proposed. Several studies suggested that agri-food wastes could be exploited as a source of proteins, polysaccharides, dietary fiber, flavor compounds, and phytochemicals as nutritional and functional ingredients (Baiano 2014). Several studies performed in recent years have pointed out that MAE represents a powerful alternative to conventional extraction methods for recovery of proteins from agri-food waste (Table 9.4).

Bran is one of the major underutilized by-products of cereal milling, which is a good source of high-quality proteins (11–15%) due to their notable levels of EAA (Skendi et al. 2020). Rice bran has received the foremost attention regarding protein extraction in last years. The extraction of proteins from rice bran is difficult due to aggregation and disulfide cross-linking (Phongthai et al. 2016). A number of studies have reported the advantages of MAE for the recovery of rice bran proteins due to the disruption of hydrogen bond networks by this physical treatment. In this context, Bandyopadhyay et al. (Bandyopadhyay et al. 2012) applied MAE (800 W for 20–90 s) for producing protein isolates from defatted rice bran meal (Table 9.4). MAE for 40 s conducted to the highest protein recovery (78%), which was notably higher than that produced by conventional boiling extraction for 1 min (36%). Longer MAE time reduced protein recovery due to their denaturation. The protein recovery was further enhanced (83%) by homogenization treatment (10 min) performed after MAE application. In a more recent study, Phongthai et al. (Phongthai et al. 2016) used the RSM to determine the optimal MAE operating conditions (microwave power, extraction time and solid-to-liquid ratio) for protein recovery from defatted rice bran. Results showed that the optimal conditions were microwave irradiation at 1000 W for 90 s and a solid-to-liquid ratio of 0.89 g rice bran/10 mL of distilled water. The application of MAE under optimal conditions

Table 9.4 Application of microwave-assisted extraction for recovery of proteins from different agri-food by-products

By-product	Solvent used	Ratio (solid/solvent)	MAE optimal conditions	PC/PR/PY	Ref.
Akebia trifoliata (Thunb.) seeds	Distilled water	1:10 w/v	500 W, 40 °C, pH 10.0, 1 h	PC: 71.50%; PY: 22.93%	(Jiang et al. 2021)
Cocoa bean shell	Deionized water	0.04 g/mL	500 W, 97 °C (heating rate: 20 °C/min), 400 rpm, 5 min	PC: 580 mg BSA/ g dm	(Mellinas et al. 2020)
Coffee silverskin	0.6 M NaOH and 0.6 M HCl (sequential extraction)	1:40 w/v	434.7 W, 10 min	PY: 43.53%	(Wen et al. 2020)
Jackfruit leaves	0.5 M NaCl	v	1200 W, 4 min	PC: 87.6 mg/g	(Moreno-Nájera et al. 2020)
Pineapple peel	Distilled water	1:8 g/mL	100 W, 8.99 min	PC: 4.0 mg/mL	(Mala et al. 2021)
Pumpkin seeds	28% w/w aqueous PEG 200-based DES	28 g/mL	140 W (microwave power), 240 (ultrasound power), 43 °C, 4 min	PY: 93.95%	(Liu et al. 2017)
Rapeseed cake	Deionized water	1:10 w/v	900 W, 30–70 °C, 45 s, pH 7.4	PY: 5–6 g/100 g dm	(Boukroufa et al. 2017)
Rice bran	Distilled water	1:10 w/v	800 W, 2450 MHz, 40s, pH 8.0	PR: 78.4% (MAE) PR: 82.5% (MAE + homogenization)	(Bandyopadhyay et al. 2012)
Rice bran	Distilled water	1:10 w/v	1000 W, 90 s, 2450 MHz, pH 10.0	PR: 22.07%; PY: 4.37%	(Uraipong and Zhao 2018)
	Distilled water	0.5:10 w/v	350–400 W, 40 °C, 90 s	PC: 75.8%; PY: 11.8%	(Bedin et al. 2020)
	Distilled water	0.42 w/v	100.7 W, 100.7 s	PY: 36 mg BSA equivalents/mL	(Hayta et al. 2021)

(continued)

Table 9.4 (continued)

By-product	Solvent used	Ratio (solid/solvent)	MAE optimal conditions	PC/PR/PY	Ref.
Sesame bran	Deionized water	1:10 w/v	750 W, 51 °C, 29 min	PY: 60.7%	(Görgüç et al. 2020)
			1.94 AU/100 g of alcalase, 750 W, 49 °C, 98 min	PY: 90.2%	
Sunflower cake	Ethanol 70%	1:5–1:15 w/v	200 W, 30 s	PC: 26%	(Náthia-Neves and Alonso 2021)
Watermelon seeds	Deionized water	1:30 w/v	50 W, 2 min, pH 10.0	PR: 90%	(Behere et al. 2021)

2-HEAA 2-hydroxyethylammonium acetate, *2-HEAF* 2-hydroxyethylammonium formate, *BSA* bovine serum albumin, *DES* deep eutectic solvent, *PC* protein concentration, *PEG*: polyethylene glycol, *PR* protein recovery, amount of protein extracted per total amount of proteins, *PY* protein yield, amount of protein extracted per mass of feedstock, *TS* total solids

enhanced protein extractability by 33% compared to the traditional alkaline extraction method, but protein digestibility remained the same. MAE (350–400 W, 40 °C, 90 s) was also successfully applied for obtaining protein extracts (75.8 g/100 g) from rice bran using shorter times compared to the conventional alkaline extraction (Bedin et al. 2020). In a recent study, Hayta et al. (2021) reported that the optimal MAE parameters for the extraction of proteins from rice bran were 100.7 W for 100.7 s, and a solid-to-liquid ratio of 0.42, in which a protein yield of 36 mg bovine serum albumin (BSA) equivalents/mL was obtained. Bran is also a by-product of sesame processing during dehulling step that contains around 15% of protein on dry weight basis. The recovery of sesame bran proteins by MAE and microwave-assisted enzymatic extraction techniques were explored by Görgüc et al. (Görgüç et al. 2020). The effect of process temperature (25–55 °C), time (10–120 min) and Alcalase enzyme concentrations (0.12–2.40 Anson units (AU)/100 g) were examined using RSM. Protein yields ranged from 44 to 62%, depending on MAE conditions and higher temperatures and longer times conducted to the highest protein recovery. Compared to MAE, higher protein yields were achieved using equivalent process time and temperature when combined extraction of MAE and enzyme addition was used. The optimal process conditions found were temperature of 51 °C and time of 29 min for MAE and enzyme concentration of 1.94 AU/100 g, temperature of 49 °C and time of 98 min of MAE combined with Alcalase treatment.

Fruit peels are another low-value by-product that may be potentially used as a valuable source of proteins, peptides, and proteolytic enzymes. Extracts containing 1.3–2.6 mg/mL of protein were produced from pineapple peels by using MAE at different power (100–300 W), irradiation time (5–15 min) and sample-to-solvent ratio (1:8–1:12 g/mL) (Mala et al. 2021) (Table 9.4). Under the optimized extraction conditions (100 W, 8.99 min and 1:8 g/mL), it resulted in an extract composed by total sugar and protein of 15.7 mg/mL and 4.0 mg/mL, respectively. Bromelain, a cysteine protease with numerous applications in food industry (meat tenderizing, brewing, and baking) was the major protein in the obtained extract.

Cocoa and coffee bean by-products have also been studied as valuable sources of proteins. Cocoa shells, the main waste obtained from the cocoa processing industry, were used for obtaining an antioxidant extract rich in proteins (580 mg BSA /g), polysaccharides (370 mg Glu/g), and phenolic compounds (35 mg gallic acid equivalent/g) (Mellinas et al. 2020) by using MAE technique (Table 9.4). The effect of pH, time, temperature, and solid-to-liquid ratio in extraction efficiency were evaluated with the aim of maximizing the yield of bioactive compounds. The results of the study showed that pH was the factor with the strongest influence on extraction yield, followed by temperature. Higher pH and temperatures were conducted to the largest protein content in the extract, and the highest protein content was observed at pH 12.0 (580.0 mg BSA/g dry matter). In another study, Wen et al. (2020) evaluated the feasibility of MAE as a green-technology to enhance protein extraction from *coffee silverskin*, a thin integument of the outer layer of coffee beans that represents a by-product of coffee roasting process. Sequential alkali-acid MAE extraction conducted to the highest protein recovery (44%) compared to conventional alkali-acid (6.2%) and UAE (14.0%) methods (Table 9.4).

Leaves from fruit trees are also agri-food wastes usually discarded that represent a suitable material to be used as a source of bioactives and nutrients. Recently, MAE conditions were optimized to recover proteins from Jackfruit (*Artocarpus heterophyllus* Lam.) leaves (Moreno-Nájera et al. 2020). Different extraction solvents (0.5 M NaCl, 96% ethanol and absolute methanol) and process times (2–4 min), as well as the effect of the addition of CH_3COONa addition (1–3%) during extraction on protein recovery, were investigated. Employment of NaCl as solvent during microwave irradiation at 1200 W for 4 min were considered the optimal conditions to maximize the protein extraction (87.6 mg/g) (Table 9.4).

Fruit seeds are a common by-product during fruit processing juices and fruit cocktails elaboration that have been scarcely used for protein recovery although they contain excellent levels of this nutrient. A novel and rapid ultrasound-microwave synergistic extraction method using a deep eutectic solvent (PEG 200/choline chloride) was developed for the extraction and recovery of proteins from pumpkin seeds (Liu et al. 2017), a waste stream generated during pumpkin processing (Table 9.4). The optimization of process parameters using Box-Behnken design determined that the optimal conditions were 28% of PEG 200-based deep eutectic solvent, a solid-to-liquid ratio of 28 g/mL, a microwave power of 140 W and a temperature of 43 °C. Under optimal conditions, a higher protein extraction yield (94%) was observed as compared with UAE and MAE processes using lesser solvent volume and shorter extraction time. A study performed by Behere et al. (Behere et al. 2021) examined different MAE parameters including sample-to-solvent ratio (1:10 and 1:40), pH (7.0–10.0), microwave power (30–70 W) and irradiation time (30 s-8 min) for the extraction of watermelon seed proteins. The results revealed that the maximum protein recovery (90%) was achieved at microwave irradiation of 50 W for 2 min, pH 10.0 and sample-to-solvent ratio of 1:30. The authors underlined that the protein recovery after MAE was enhanced with a drastic reduction of solvent volume and process time as compared to conventional alkaline and UAE extractions. Watermelon seed proteins obtained by MAE exhibited better in vitro digestibility than those obtained by the other extraction techniques. Likewise, seeds from *Akebia trifolata* (Thunb.), a waste derived from the oil processing extraction of the fruit in China were investigated for obtaining protein isolates by applying MAE as pretreatment of enzymatic hydrolysis (Jiang et al. 2021). MAE followed by cellulase hydrolysis increased protein yield (22.93%) compared with conventional sequential alkaline-acid extraction (17.1%), enzymatic-assisted sequential alkaline-acid extraction (19.4%), ultrasound-assisted enzymatic-assisted sequential alkaline-acid extraction (20.8%) and shear emulsifying-assisted enzymatic-assisted sequential alkaline-acid extraction (21.4%).

Studies aimed at valorizing *oil refining industry by-products* as a source of proteins have been recently conducted. In this scenario, MAE was applied to produce a flour with high protein content (26%) from sunflower cake, a by-product of sunflower oil processing, rich in proteins (27–63%) (Náthia-Neves and Alonso 2021) (Table 9.4). The authors optimized solvent concentration, solvent to sample ratio, microwave irradiation power (100–300 W) and process time (30–120 s) for obtaining an extract rich in chlorogenic acid and a residual solid

(flour) rich in proteins (26%) with high content of EAA that makes it a valuable food product for human nutrition. Similarly, the protein-rich cake resulting after the extraction of rapeseed oil has been indicated as a good alternative protein source for human nutrition due to its balanced amino acid profile and techno-functional attributes. Thus, great interest has focused on optimizing the extraction process of protein from this by-product. Boukroufa et al. (2017) investigated the protein recovery efficiency of three innovative extraction methodologies including UAE, MAE, and percolation, in comparison with that obtained by conventional extraction. Results of this study demonstrated that although UAE resulted in the highest protein yield (8.1 g/100 g), MAE also enhanced the protein extraction (5–6 g/100 g depending on temperature) due to the microwave thermal effect responsible for the disruption of cell walls and tissues which help soluble protein to disperse.

9.6 Conclusion and Future Perspectives

Protein extraction is considered as a critical step in contributing to the final greenness of the complete protein analysis process. In the last years, important advances have been accomplished with the design, development, and application of novel methodological approaches for extracting high-quality proteins at high yields, reducing the use of toxic chemicals, as well as the temperature and time conditions of the extraction process. Therefore, emerging eco-innovative extraction technologies are becoming a promising alternative to conventional methods for recovering safe and nutritive proteins with preserved techno-functional properties from novel sources. Among these extraction technologies, MAE has a great potential for process intensification in the separation and purification of proteins and could promote new opportunities for food innovation. In MAE, when raw materials are exposed to microwave irradiation, the fast superheating and evaporation of water accelerate the release and extraction of proteins. These features of microwave energy are behind a wide range of advantages such as the enhancement of process efficiency, the increase of protein yield, and the reduction of process time, costs, energy, and solvent consumption and thus, the decrease of the environmental impact. These advantages have made MAE to be successfully applied for the extraction of proteins from plant sources. The outcomes of this approach are influenced by the operating factors and features of the plant matrix, thus, the selection of appropriate solvents and identification of optimal exposure time, temperature, and microwave power for each food matrix is crucial to achieve the highest yield. Despite being considered a promising eco-innovative methodology to extract high-quality proteins from plant sources, to date, the research on MAE is still in its infancy and most of the studies have been conducted at laboratory scale. Thus, further studies demonstrating the economic and environmental suitability of implementing MAE within the existing processing lines at industrial scale are required.

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Micellar Precipitation and Reverse Micelle Extraction of Plant Proteins

10

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Abstract

Current food challenges have allowed the development of innovative technologies to supply the global protein demand. Plant-based proteins are the most convenient protein sources due to their techno-functional properties, such as solubility, viscosity, emulsification, and water and oil retention capacity. Isoelectric precipitation is the most popular method to produce protein concentrates/isolates; however, other technologies, such as micelle precipitation and reverse micelle extraction, are underutilized technologies in the protein ingredients industry, despite their multiple advantages over other techniques, such as low denaturation of native plant proteins. Micelles, as nano-structures, are formed by amphipathic molecules with the polar heads in contact with the surrounding solvent, whereby hydrophobic chains of the micelle orient themselves inward. Proteins can be recovered easily from water-matrixes during micelle formation and can be precipitated, purified, and processed for multiple purposes. Reverse micelles are nano-sized aggregates of surfactant molecules in nonpolar organic

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solvents, containing an inner core of water molecules. During reverse micelle extraction, the proteins are attracted into the inner water core of the reverse micelles and they can then be recovered. Despite the few studies available about these extraction processes, the literature supports the fact that micellar precipitation and reverse micelle extraction may represent economic alternatives to obtain proteins at an industrial scale from oilseeds, pseudocereals, cereals, and legumes, as well as unexplored non-animal food sources.

Keywords

Micellar precipitation · Reverse micelles · Plant-based proteins · Green protein processing technologies

10.1 Introduction

In recent years, public health awareness has improved, especially regarding food security and the availability of healthy and nutritious food. The global food system provides billions of people with access to affordable, safe, and nutritious food for a healthy diet and protein to support nutritional requirements. However, it is as well one of the major sources of greenhouse gas emissions, depleting natural resources, and failing to secure healthy diets for all communities around the world, thus requiring urgent transformational changes in the food system. Due to climate change and the growing world population, consumers are switching to more environmental-friendly, nutritious, and sustainable foods, thus increasing the use of plant protein sources to substitute and/or reduce animal protein consumption by adopting plant-based and flexitarian diets (Sá et al. 2020). Animal protein shows increased financial costs and limited supply, which has been proven to be highly related to climate change, freshwater depletion, biodiversity loss, and hazards to human health (Alemayehu et al. 2015; Pojić et al. 2018). Based on its wide diversity in nature and consumers' pursuit of healthy ingredients, the demand for plant protein is increasing at a staggering pace (Timilsena et al. 2016).

Proteinaceous seeds from different plant sources have shown a large number of health-promoting effects, including prevention of cardiovascular diseases such as type 1 and 2 diabetes, blood cholesterol and triglyceride level lowering properties, anticancer, anti-inflammatory, and antioxidant among others (López et al. 2018; Guyomarc'h et al. 2021; Sucher et al. 2017; Li et al. 2017; Wen et al. 2020; Chalamaiah et al. 2018). Several plant sources have been studied and used as a protein supplement, such as pulses (pea, bean, chickpea, lupin, faba bean, cowpea) (Nishinari et al. 2014; Burger and Zhang 2019), cereals (rice, wheat, millet, sorghum, maize, and barley) (Fabian and Ju 2011; Voci et al. 2020), pseudocereals (amaranth, quinoa, and buckwheat) (Dakhili et al. 2019; López et al. 2018), oilseeds (chia, flaxseed, sesame, pumpkin, soybean, and sunflower) (Sandoval-Oliveros and Paredes-López 2013; Parikh et al. 2019), as well as almonds and nuts (Fernandes et al. 2010). Depending on the source and extraction process, plant proteins display

different physicochemical and techno-functional properties. However, the extraction process of plant proteins affects their functional properties, molecular weight, molecular structure, and charge density. Plant proteins still present several challenges for industrial upscale such as optimization of extraction processes, organoleptic constraints (flavor, color, and taste), reduced digestibility, presence of limiting amino acids, among others. However, their use in food processing is considered a sustainable solution due to their abundance in nature, low cost of production, health benefits, and low environmental impact (Pojić et al. 2018).

The major protein fractions of pulses include globulins accounting for 70–90% and albumins ranging between 10 and 30% (Sharif et al. 2018). Depending on their sedimentation coefficients, globulins consist of two types known as 7S globulins and 11S globulins (Sha and Xiong 2020). While albumins have a sedimentation coefficient of 2S. Globulins are high molecular weight proteins (8–600 kDa) with poor solubility in water but soluble in salt solution, while albumins are low molecular proteins (5–80 kDa) highly soluble in aqueous media (Burger and Zhang 2019). The ratio between globulin/albumin is affected by varietal characteristics, growth environmental conditions, extraction process, among others (Sharif et al. 2018).

Proteins with various physicochemical properties are extracted from major cereals such as maize, wheat, millet, and rice. There are different groups of cereal proteins including prolamins, albumins, globulins, and glutelins (Muhoza et al. 2021). The high percentage of storage proteins in cereals is made up of prolamins 20–50% and globulins 20–50%, respectively. These proteins are characterized by their hydrophobic nature and poor solubility in water. Albumins are soluble in water, globulins are salt soluble, glutelins are soluble in alkali/acid solution, and prolamins are soluble in alcohol solution. The solubility of these proteins is influenced by surface composition, charge, and intermolecular interactions (Muhoza et al. 2021). The molecular weight of cereals proteins ranges from 10 to 100 kDa for prolamins, 60 to 120 kDa for globulins, 30 to 40 kDa for low molecular weight glutelins and 12 to 30 kDa for albumins (Sha and Xiong 2020).

Another common source of protein is oilseeds which have various techno-functional properties and well-balanced amino acid composition (Chmielewska et al. 2020). The physicochemical properties of oilseed proteins are affected by the source, climate condition, geographic location, extraction and isolation processes. Oilseed proteins are composed of nearly 70% of globulins/cruciferins and 30% albumins/napins. The molecular weight of cruciferins can vary from 20 to 200 kDa while the molecular weight of napins ranges between 50 and 200 kDa (Muhoza et al. 2021).

The most widely applied protein extraction process at an industrial scale is the alkaline extraction-isoelectric precipitation process. It consists of the extraction of the proteins at alkaline pH (usually between 8 and 11) under agitation, followed by the removal of the insoluble matter by centrifugation, and by the isoelectric precipitation of the proteins contained in the supernatant by the addition of acids to decrease the pH to the isoelectric point of the proteins, which is around 4.5 for most plant proteins. A centrifugation step is then carried out to recuperate the precipitated proteins that are resolubilized in water at pH 7 before being dried to obtain a protein

concentrate or isolate (Mondor and Hernández-Álvarez 2022). The main advantage of the alkaline-isoelectric precipitation process is its high productivity and its scalability. However, it also has some limitations such as the large amount of effluents that are generated and which can result in a negative impact on the environment when they are not treated properly. It is also known that harsh chemicals are used in this process, thus having a negative impact on the functionality of the proteins, especially the protein's solubility after rehydration (Mondor et al. 2004). Consequently, various alternative processes have been considered for the extraction/purification of plant proteins including micellar precipitation (Mondor and Hernández-Álvarez 2022) and reverse micelle processing.

10.2 Micellar precipitation and Reverse micelle Extraction

10.2.1 Micellar Precipitation

Micelles are nano-sized aggregates that form in water with the polar heads in contact with the surrounding solvent, whereby hydrophobic chains of the micelle orient themselves inward (Fig. 10.1) (Sankaran et al. 2019). Micelle formation helps to attract proteins that are solubilized in the water phase and under certain conditions these can be precipitated and then recovered. The formation of micelles and their precipitation has been applied in the food industry to isolate and recover plant proteins (Abdel-Aal et al. 1986; Cordero-de-los-Santos et al. 2005; Dapčević-Hadnađev et al. 2019; Hadnađev et al. 2018; Krause et al. 2002; Lampart-Szczapa 1996; Paredes-Lopez and Ordorica-Falomir 1986; Paredes-Lopez et al. 1991; Stone et al. 2015). During the first step, plant proteins are extracted in a salt solution followed by a centrifugation step to remove the insoluble matter. The extraction step

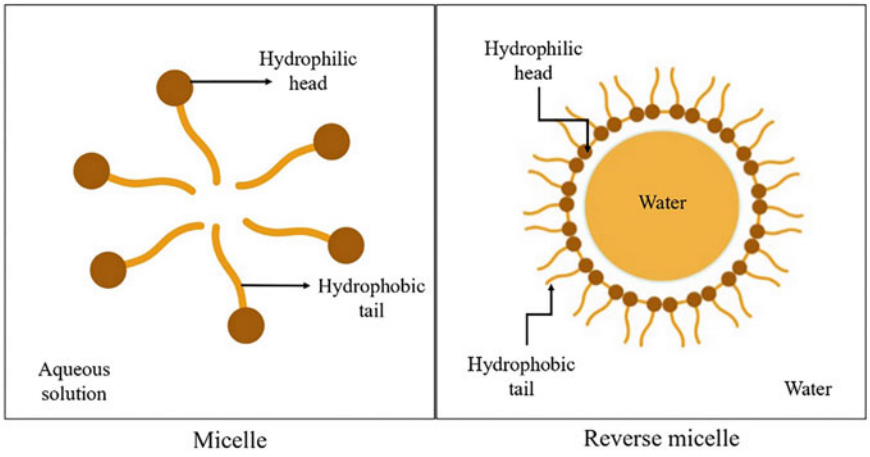

Fig. 10.1 Micelle and reverse micelle

is followed by a precipitation step to recover the proteins in a micelle-like form that is stabilized by hydrogen bonds that contains both globulins and albumins. In order to precipitate the proteins, cold water is usually added at a ratio of high-salt protein extract to water of 1:3 to 1:10 (v/v) which results in the formation and precipitation of micelles. Micelle formation can be maximized by leaving the diluted solution to stand for a time prior to the centrifugation step, thus enhancing the recovery of precipitated micelles before drying.

When compared to the conventional isoelectric precipitation process, the main advantage of the micellar precipitation process for the recovery of plant proteins is its milder conditions which result in less protein denaturation and better functional properties. However, the protein recovery is low when compared to the conventional alkaline solubilization coupled with isoelectric precipitation process (Cordero-de-los-Santos et al. 2005).

10.2.2 Reverse Micelles

Reverse micelles (RMs) have become more popular during the last decade due to their wide range of applications such as extraction of food proteins, simultaneous extraction of oils/proteins, purification and/or extraction of enzymes, and enrichment of components such as amino acids for analysis (Table 10.1). Nowadays, they have a new application as nutraceuticals for delivering functional ingredients due to their nanocarrier structure. RMs can be described as nano-sized aggregates of surfactant molecules in nonpolar organic solvents, containing an inner core of water molecules (Sankaran et al. 2019; Zhu et al. 2009). The surfactant molecules consist of a polar head and a nonpolar tail structure. Being hydrophilic, the polar heads orient themselves toward the water content at the inner core, whereas the apolar or hydrophobic tails orient themselves toward the organic solvent as shown in Fig. 10.1 (Pojić et al. 2018; Sankaran et al. 2019). RMs may have different characteristics such as size, shape, aggregation number, internal structure of cores, and microviscosity (Bu et al. 2014; Sankaran et al. 2019; Sun and Bandara 2019). Anionic, cationic, zwitterionic, nonionic, and mixed surfactants can be used for the preparation of RMs (Sun and Bandara 2019). Sugar surfactants can also be used; they are relatively new non-toxic and environmentally friendly, and their chemical composition is a sugar head group and alkyl chain. Sugars can be of natural origin or can be synthesized chemically or enzymatically (Pojić et al. 2018). The overall RM structure can be defined as a water-in-oil structure.

RM can be characterized based on its water content and its aggregation number. The water content (W_0) represents the number of water molecules per surfactant molecule. The values of W_0 can reach values as large as 40–60, the RM radius corresponds to the water content of the micelle (Sankaran et al. 2019; Sun and Bandara 2019). In general, the size of RMs ranges from less than 1 nm to approximately 14 nm. The size of RMs is influenced by the addition of water to the system. The addition of extra water to the system will result in an increase in the size of RMs. The equation to calculate the W_0 is:

Table 10.1 Composition of reverse micelle systems (modified from Sun and Bandara 2019)

Surfactant name	Surfactant type	Nonpolar solvent
AOT	Anionic	Isooctane
CTAB	Cationic	Methenyl trichoride and butyl alcohol
Gemini surfactant	Cationic	n-hexane and 1-hexanol
L-proline propyl ester lauryl sulfate	Anionic	Cyclohexane
AOT/tween 85	Mixed surfactants	Isooctane and n-octyl alcohol
Phosphatidylcholine	Zwitterionic	Oil
DOPC or DOPE	Zwitterionic	Oil
DTAB	Cationic	Isooctane
CTAB	Cationic	Isooctane
CTAB	Cationic	Isooctane and hexylalcohol
Span 20	Nonionic	Isooctane
3,3-dymethyl-1-butylsulfosuccinate sodium salt	Anionic	Pentane
A new amphiphilic homopolymer	Cationic	Toluene
Triton X100	Nonionic	n-hexane
PEG-b-PLA or PEG-b-PLA-CD	Amphiphilic	Dichloromethane and ethyl oleate
Phosphatidylcholine	Zwitterionic	Tertiary butyl alcohol
PEG-b-PLA	Amphiphilic	Toluene
Monoglycerides	Nonionic	Oil and ethanol

AOT Sodium bis (2-ethylhexyl) sulphosuccinate; *CTAB* ceryl-trimethyl-ammonium bromide; *DOPC* dioleoyl phosphocholine; DOPE: dioleoyl phosphoethanolamine; *DTAB* dodecyl trimethyl ammonium bromide; *PEG-b-PLA* polyethylene glycol block polylactide; *PEG-b-PLA-CD* polyethylene glycol block polylactide and β-cyclodextrin

$$W_0 = \frac{\text{Water}}{\text{Surfactant}}$$

Another parameter used to characterize RM is the aggregation number (N_{ag}) which is the average number of surfactant molecules in each RM. When the hydrocarbon chains of surfactant increase, the N_{ag} will also increase. The N_{ag} is calculated using the following equation:

$$N_{ag} = \frac{\text{Spherical surface area of reverse micelle}}{\text{Surface area of surfactant headgroup}}$$

Sankaran et al. (2019) identified three processes that can be carried out for the purification and recovery of proteins by RMs formation, namely the incorporation of proteins into RMs by the injection method, by dry addition method, and the most commonly used by phase transfer. In the injection method, the proteins are first solubilized in the aqueous phase which is then added into a mixture of organic solvent and surfactant which is mixed under high agitation. The dry addition method

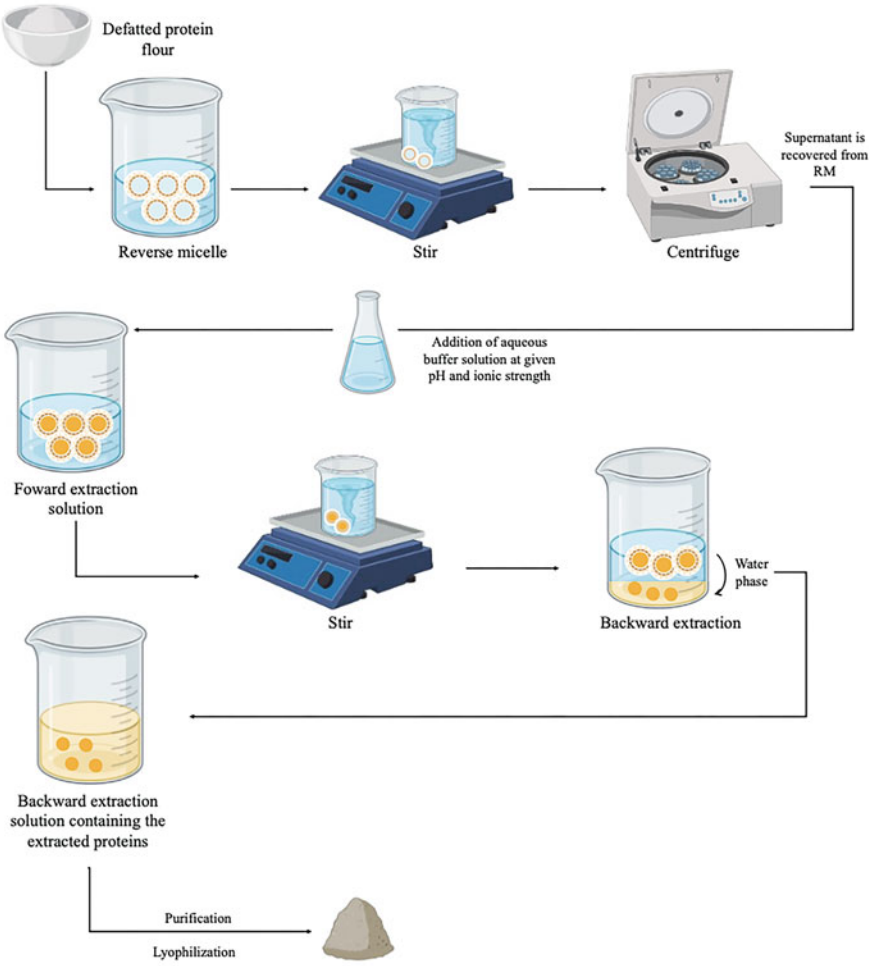

Fig. 10.2 Reverse micelle extraction of proteins

is similar to the injection method; however, the proteins are added to the RMs comprising the organic phase in the form of a dry powder instead of being added as an aqueous phase. The dry proteins are dissolved into the system under high agitation. However, the most commonly used process is the phase transfer process, which consists of a two-step process known as the forward and backward extraction step (Fig. 10.2). In the forward extraction step, the first stage consists of the formation of the RMs, followed by the incorporation of the proteins into the inner water core of the RMs and the recuperation of the RMs containing the proteins by phase separation. In the backward extraction step, the proteins are extracted from the RMs into a new water phase which increases the purity of the recovered proteins. The efficiency of the forward extraction step is affected by many experimental

parameters such as the type and concentration of surfactant molecules, the type and structure of solvent, pH of the aqueous phase, ionic strength, type of salt, protein charge, temperature, water content, and the size and shape of the RMs (Sankaran et al. 2019). The pH and temperature of extraction, the type and concentration of salt, surfactants, protein, and the concentration of the stripping solution are the main parameters affecting the efficiency of the backward extraction.

In the food industry, RMs have been extensively used to isolate and recover plant proteins (Bu et al. 2014; Chen et al. 2014; Liu et al. 2014; Pojić et al. 2018; Sun et al. 2008, 2009; Wang et al. 2021; Zhang et al. 2017, 2018; Zhao et al. 2010, 2015a, b, 2018; Zhu et al. 2009, 2010). The phase transfer process is the most used for the purification and recovery of plant proteins.

As with any method, RM has advantages and disadvantages. Among its advantages, RM is easy to implement (Sankaran et al. 2019). The solvent can also be recovered which diminishes the cost of the technique. Additionally, the core in RM is polar, allowing the solubilization of the proteins without affecting their native conformation or changing their activity (Chen et al. 2014; Pojić et al. 2018). However, despite the great potential of RM extraction for the recovery of plant proteins, some limitations remain such as the fact that the most widely used system consists of sulphosuccinic acid bis (2-ethylhexyl) ester sodium salt (AOT) and isooctane, two chemical compounds that are not approved for food use. Therefore, there is a need to find biocompatible and edible surfactants and apolar solvents for the development of efficient food-grade RM extraction systems. Also, RM extraction still has a limited productivity and a high cost when compared to the conventional isoelectric precipitation process used to recover plant proteins. Although it is claimed that RM is convenient and easy to scale up, RM extraction has been applied at laboratory scale only, and the scale-up of the technology remains a challenge (Pojić et al. 2018).

10.3 Impact of each Technology on the Protein Recovery and Purity for Different Plant Sources

10.3.1 Micellar Precipitation

Micellar precipitation is a method performed for protein extraction from several food matrices, including plant-based protein sources (Abdel-Aal et al. 1986; Paredes-Lopez and Ordorica-Falomir 1986; Paredes-Lopez et al. 1991; Krause et al. 2002; Tanger et al. 2020). Micellar precipitation protocols applied for the isolation of plant-based proteins are summarized in Table 10.2.

Abdel-Aal et al. (1986) explored three methods to isolate proteins from three pulses. After applying isoelectric precipitation, micellar precipitation, and partially hydrolyzed protein precipitation, the protein content of the resulting ingredients varied considerably, with faba bean-derived ingredients showing values ranging between 69.6 and 86.3%, fenugreek between 59.3 and 62.4%, and chickpea between 59.5 and 81.5%. The highest protein yield was observed by isoelectric precipitation

Table 10.2 Protein extraction by micellar precipitation of some plant-based sources

Micellar Precipitation						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein yield	Main Findings	Reference
Faba bean (FB, *Vicia faba*), chickpea (CP, *Cicer arietinum*) and fenugreek (FG, *Trigonella foenum-graecum*)	1) IP (alkaline extraction and precipitation at isoelectric pH) 2) MP (extraction in 0.5 M sodium chloride solution and precipitation by ionic strength reduction) 3) HP (extraction in the presence of pepsin or pancreatin)	Flours (g/100 g dw) (N × 5.85): FB 30.7, CP 25.8, FG 30.7	IP (g/100 g dw): FB 75.2, CP 59.5, FG 60.4 MP (g/100 g dw): FB 86.3, CP 81.5, FG 59.3 HP (g/100 g dw): FB 69.6, CP 60.8, FG 62.4	IP (g/100 g dw): FB 66.2, CP 53.0, FG 30.8 MP (g/100 g dw): FB 74.0, CP 56.0, FG 24.3 HP (g/100 g dw): FB 63.9, CP 52.5, FG 28.2	• oil emulsification property of the isolate was MP < HP < IP • PHP had the highest nitrogen solubility index (NSI) at pH 2.4; MP at pH 5.7–6.4; IP at pH 4.5–5.5	Abdel-Aal et al. (1986)
Amaranth (*Amaranthus hypocondriacus*)	1) IP 2) MP	–	IP: 93.1% MP: 80.2%	IP: 56.4% MP: 15.9%	• Peptide of 1380 kDa was found in PIP concentrates; 905 and 190 kDa peptides were found in MP. • MP showed higher denaturation enthalpy suggesting homogeneous composition and less denatured protein composition. • Foaming and	Cordero-de-los-Santos et al. (2005)

(continued)

Table 10.2 (continued)

Micellar Precipitation						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein yield	Main Findings	Reference
					emulsification properties were better at acidic pH for both IP and MP.	
Hemp (*Cannabis sativa*)	1) IP 2) MP	–	IP: 40.2% MP: 50.6%	IP: 98.9% MP: 91.4%	• Solubility at pH 3: IP 71.2%, MP 88.2%. • MP expressed slightly higher surface/interfacial activity than IP.	Dapčević-Hadnađev et al. (2019)
Hemp seed meal (*Cannabis sativa*)	1) IP 2) MP	–	IP: 40.2% MP: 50.6%	IP: 98.9% MP: 91.4%	• IP exhibited lowest protein solubility at pH 5.0, while for MP it was shifted to pH 6.0. • Protein conformational stability of MP showed lower water retention capacity.	Hadnađev et al. (2018)
Flaxseed (*Linum usitativissimum*)	1) MP 2) IP	Flaxseed flour: 50%	MP 93% IP: 89%	–	• 11S globulin (linin) was the main protein fraction in both isolates • Protein stability of MP was ~90% at pH	Krause et al. (2002)

					values used in techno-functional properties studies.	
Safflower (*Carthamus tictorius*)	Extraction 10% (NaCl), pH 5.8 and 7.0, for 30 min at 35 °C; centrifuged 5000 × *g*, 15 min, supernatant diluted in deionized water (1:4). After standing for 30 min at room temperature, the protein was recovered by centrifugation at 10,000 × *g* for 20 min and freeze-dried.	Meal prepared at laboratory (N × 5.3): 46.8 g/100 g Meal prepared at industrial scale (N × 5.3): 28.2 g/100 g	SML-MP: 84.8%; SML-IP: 85.2%; SMI-MP: 97.4%; SMI-IP: 85.4%	SML-MP: 44.2% SML-IP: 78.8% SMI-MP: 17.2% SMI-IP: 46.9%	• Mild conditions used in the MP procedure may allow the protein to retain its native structure (hydrophobic interactions are known to play a major role in the stabilization of this isolate). • MP concentrate/ isolate had higher solubility. • All concentrates/ isolate had a similar amino acid composition and in-vitro protein digestibility.	Paredes-Lopez and Ordorica-Falomir (1986)
Chickpea (*Cicer arietinum*)	For the micellization procedure, 0.5 M NaCl, pH 7.0 was used to extract proteins from defatted flour (10%, w/v). A membrane cartridge with a molecular weight cut-off of 10 kDa was used for concentration.	Chickpea flour: 17.2 (g/100 g dw) (N × 6.25)	MP: 87.8 g/ 100 g IP: 84.8 g/ 100 g	–	• Higher denaturation temperature and transition enthalpy in MP. • SDS-PAGE showed a molecular weight distribution between 16.6 and 66.4 kDa for MP and 14.9 and 84.2 kD for IP proteins.	Paredes-Lopez et al. (1991)

(continued)

Table 10.2 (continued)

Micellar Precipitation						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein yield	Main Findings	Reference
	Proteins were flocculated by adding water at 4 °C (1:4 v/v, protein extract:Water) at pH 7.0				• Most essential amino acids of chickpea isolates were at acceptable levels compared to a reference pattern (casein).	
Field pea (*Pisum sativum*) market classes striker, meadow and Dakota	For MP, 50 g of defatted pea flour was suspended in a 1.0 N NaCl solution at a 1:10 (w/v) ratio and stirred (500 rpm) for 2 h at room temperature. After centrifugation at 4000 × *g* for 20 min at 4 °C, the supernatant was collected and diluted tenfold with cold deionized water (4 °C), and then left for 18 h at 4 °C. The solution was then centrifuged again at 4000 × *g* for 20 min at	–	IP: 83.3–86.9% SE: 71.5–79.3% MP: 81.9–87.8%	IP:62.6–76.7% SE: 68.2-74.8% MP: 30.7–31.1%	• SE and IP (~70%) resulted in higher protein recoveries than MP (~31%). • MP had the lowest surface hydrophobicity. • Salt-extracted isolates exhibited the highest protein solubility (~89%) and MP isolates the lowest (~46%). • Salt-extracted isolates had the highest oil holding capacities (5.3 g/g) and the lowest water holding capacities (0.3–2.6 g/g).	Stone et al. (2015)

	4 °C. The pellet was collected and stored at −30 °C until freeze-dried.				• All isolates displayed high emulsion stability (~98%).	
A commercial PPI E86 with 70.8% protein on dry basis from Emsland Starke (Emlichheim, Germany)	MP were obtained from 50 g pea flour dispersed in 500 g, 1 M NaCl solution (1:10 w/w) and stirred for 2 h at 20 °C at 500 rpm. The mixture was centrifuged at 4500 × *g* for 20 min at 4 °C. The pellet was discarded, and the supernatant was diluted tenfold with water and then left for 18 h at 4 °C. The dispersion was centrifuged at 4500 × *g* for 20 min 4 °C. The pellet was collected, stored at −80 °C and then freeze-dried.	18.27% protein on dry basis	IP:74.2–74.5% MP: 75.1% SE: 73.2%	IP: 46.0–50.0% PMP: 25.0% SE: 39.7%	• Albumins were lost during precipitation in alkali extraction–isoelectric precipitation and MP. • MP protein product formed micelles at low ionic strength and solubility of these could be increased by addition of salt.	Tanger et al. (2020)

IP isoelectric precipitation; *MP* micellar precipitation; *HP* hydrolyzed precipitation: ↑ increase; ↓ decrease; *SML* Laboratory-prepared safflower meal; *SMI* Commercially prepared safflower meal; *PPI* pea protein concentrate; *SE* salt-extraction dialysates

in fenugreek sample (30.8%), while micellar precipitation displayed the higher value for protein recovered in faba bean and chickpea samples (74.0% and 56.0%, respectively). For chickpea, Paredes-Lopez et al. (1991) found that micellar precipitation increased the protein content of the resulting concentrate 5.1-fold, when compared to the defatted chickpea flour. In comparison, an increase of 4.9-fold was observed for the isoelectric protein precipitation process.

For field pea defatted flour, alkaline extraction/isoelectric precipitation resulted in concentrates with similar values of protein content in comparison with micellar precipitation, with values of 83.3–86.9% and 81.9–87.8%, respectively, while salt extraction dialysis resulted in the concentrates with the lowest protein content (71.5–79.3%) (Stone et al. 2015). The corresponding protein yields were 62.6–76.7%, 30.7–31.1%, and 68.2–74.8%, respectively. Meanwhile, for a commercial pea protein isolate, micellar precipitation had the lowest protein yield (25.0%) compared with alkaline extraction/isoelectric precipitation (46.0–50.0%) and salt extraction dialysis (39.7%). However, the concentrate obtained by micellar precipitation was found to have a protein content of 75.1% compared to 73.2% for salt extraction dialysis and 74.2–74.5% for alkaline extraction-isoelectric precipitation (Tanger et al. 2020).

In another work, Krause et al. (2002) found a protein content of 93% after micellar precipitation of flaxseed (*Linum usitativissimum*) samples; however, the protein content for isoelectric precipitation showed a similar value at 89%. In amaranth (*Amaranthus hypocondriacus*), a pseudocereal, micellar precipitation was less effective than isoelectric precipitation for protein recovery with a value of 15.9% compared to 56.4%. The corresponding protein content in the resulting concentrates were 80.2% and 93.1%, respectively (Cordero-de-los-Santos et al. 2005). Similar results were observed for safflower (*Carthamus tirctorius*) samples by Paredes-Lopez and Ordorica-Falomir (1986), who reported protein yields of 17.2% and 44.2% in micelle precipitates from commercial-prepared and laboratory-prepared safflower meals, respectively, which were lower than for isoelectric precipitation (46.9% and 78.8%, respectively). The corresponding protein content in the resulting concentrates were 97.4% and 84.8%, and 85.4% and 85.2%, respectively. Hadnađev et al. (2018) reported a protein yield of 40.2% for micellar precipitation of hemp (*Cannabis sativa*) proteins, while the alkaline extraction-isoelectric precipitation process showed a value of 50.6%. The corresponding protein content in the resulting concentrates were 98.9% and 91.4%, respectively.

The protein yield is the most common parameter to estimate the effectiveness of the micellar precipitation and of other methods for protein concentration and isolation; however, other indicators may help evaluate the application potential of these techniques, such as the protein/peptide stability, solubility, or molecular weight (Abdel-Aal et al. 1986; Krause et al. 2002; Hadnađev et al. 2018; Dapčević-Hadnađev et al. 2019). High protein/peptide stability is a desirable characteristic for protein micelles formation because this decreases the water retention, enhances a homogenous protein composition, and improves the micelle solubility in hydrophilic matrices and the surface/interfacial activity (Cordero-de-los-Santos et al. 2005; Hadnađev et al. 2018; Dapčević-Hadnađev et al. 2019). The molecular weight is

another indicator of the quality of protein micelles. Some reports suggest that high molecular weight proteins are not easily integrated into the micelle structure during their assembly; thus, micelle precipitates could release easily their carrying peptides and other compounds (Bath et al. 2016; Joseph et al. 2017). Frequently, proteins above >500 kDa, such as albumins and globulins, are kept out of the micelles, and only subunits of these proteins are incorporated into the micelles (Paredes-Lopez and Ordorica-Falomir 1986). Additionally, these indicators may suggest that micelle precipitates could carry phytochemicals and other bioactive compounds, including polyphenols, metal ions, and carotenoids (Kumar et al. 2008; Cala et al. 2012; Sáiz-Abajo et al. 2013), that can be released during gastrointestinal digestion (Paredes-Lopez and Ordorica-Falomir 1986; Paredes-Lopez et al. 1991).

Several authors have declared that micellar precipitation is a practical and optimal method for protein isolation from different food matrices. Micellar precipitates have a good nitrogen solubility index at acidic pH, which can facilitate protein digestibility, at the same time the protein hydro-solubility is increased (Abdel-Aal et al. 1986; Krause et al. 2002; Dapčević-Hadnađev et al. 2019; Stone et al. 2015; Tanger et al. 2020). Micelle precipitates may also be formed by homogeneous molecular-size peptides (Cordero-de-los-Santos et al. 2005). In addition, in unfavorable heat conditions, micelles seem to keep the native structure of some proteins and peptides, suggesting they could resist extraction and isolating conditions (Paredes-Lopez and Ordorica-Falomir 1986; Cordero-de-los-Santos et al. 2005). Micellar precipitation also has the capacity of entrapping phytochemicals found in plants, such as polyphenols (Dapčević-Hadnađev et al. 2018), which have been studied for their bioactive potential promoting human health (Sánchez-Velázquez et al. 2021). Finally, micelles may present higher surface/interfacial activity than alkaline precipitates, which could represent an advantage for protein solubilization, digestion, and degradation (Dapčević-Hadnađev et al. 2018). Despite all these advantages, the micellar precipitation of plant proteins needs to be studied in more detail and to be optimized for large-scale industrial applications and food purposes.

10.3.2 Reverse Micelles

Different authors have used RM extraction for plant protein sources (Table 10.3). Sun et al. (2008) reported the optimization of the forward extraction methodology of RM. The authors extracted protein from defatted wheat germ (31.48%), using sulfosuccinate sodium salt (AOT) as a surfactant and isooctane and KCl solution as nonpolar solvents, and comparing different parameters such as pH (2.08, 4.01, 6.10, 8.22, 10.15, and 12.32), adjusted W_0 (8, 12, 17, 21, 25, and 33), amount of AOT (1.0, 2.0, 3.0, 4.0, and 5.0 g suspended in 50 mL of isooctane) and KCl solution concentration (0.1, 0.2, 0.3, 0.5, 0.75 mol/L). The highest forward extraction efficiency (37%) was reached after 30 min, with a fixed temperature of 36 °C, a pH of 8, AOT concentration of 0.06 g/mL, and KCl concentration of 0.1 mol/L. The authors concluded that the efficiency of the forward extraction decreased with an increase in AOT concentration. Another study by Sun et al. (2009) optimized a

Table 10.3 Protein recovery by reverse micelle extraction of plant protein sources

Reverse Micelle Extraction						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein Yield	Main findings	Reference
Soybean flour	Reverse micelle system: a) AOT in isooctane (4:1) and KCl-phosphate buffer. b) AOT and tween 85 (4:1) in isooctane and n-octyl alcohol (4:1). Backward extraction: Stirring (1 h; 30 °C) the upper oil phase with 0.1 M NaCl in 70% ethanol solution (pH 4) at a ratio of 2.5:1 (v/v).	42.2%	AOT: 80.2% AOT/tween 85: 79.1%	AOT: 28.0% AOT/tween 85: 29.0%	Efficiency of forward extraction of soya bean is higher using AOT/tween 85 reverse micelle system.	Bu et al. (2014)
Soybean flour	Forward extraction: AOT in hexane (90-315 mM) with added KCl solution (0.00-0.35 M), pH (5.5–9.0) and alcohol amounts (0.1–0.8 vol%). Backward extraction: The liquid phase containing the soybean proteins was added to an equivalent volume of aqueous phase containing a fixed AOT concentration, KCl concentration and pH value as well as alcohol (0.1–0.8%, v/v). The backward extraction was conducted for 60 min at 35 °C.	37.53%	–	Forward extraction: Up to 70.1% Backward extraction: Around 92%	For forward extraction optimum conditions were AOT concentration of 180 mM, aqueous pH of 7, 0.05 M KCl and 0.5% (v/v) alcohol. Phosphate buffer, KCl 1 M and 60 min.	Chen et al. (2014)

Defatted wheat germ flour	Reverse micelle with AOT in isooctane. pH (2.08–12.32), adjusted W_0 (8–33), amount of AOT (1.0–5.0 g suspended in 50 mL of isooctane) and KCL solution concentration (0.1–0.75 M).	(N × 5.45): 31.48%	–	Forward extraction: Up to 37%	The highest forward extraction efficiency (37%) was reached after 30 min, with a fixed temperature of 36 °C, a pH of 8, AOT concentration of 0.06 g/mL and KCl concentration of 0.1 M.	Sun et al. (2008)
Defatted wheat germ flour	Reverse micelle with AOT in isooctane (3:50, w/v), W_0 adjusted to 25 by 0.1 M KCL solution, with a pH of 8, for 60 min at 36 °C. Backward extraction: Recovery of the isooctane. Addition of a KCl solution (0–2.5 mL, pH 7–10 and concentration 0.25–1.25 M) to the resulting solution.	(N × 5.45): 31.48%	–	Backward extraction: 80%	Optimum conditions for the backward extraction were identified as follows: KCl concentration of 0.61 M, a pH of 9.47 and a volume of 1.0 mL.	Sun et al. (2009)
Defatted walnut flour	Forward extraction: Flour was mixed with AOT/hexane (1:50 w/w, 50 min,40 °C) followed by centrifugation. Backward extraction: The phase containing the walnut protein was added to a 1 M KCl solution followed by microwave extraction for 25, 30, and 35 min at 40, 45, and 50 °C and KCl solution –forward extraction solution ratio (1:1, 2:1, and 3:1). Followed by centrifugation at 4000 g for 10 min.	46.96%	–	Backward extraction: Up to 95.43%	Optimal conditions were found to be 30 min, 45 °C, and an aqueous solution to forward extraction solution ratio of 3:1 (v/v)	Wang et al. (2021)

(continued)

Table 10.3 (continued)

Reverse Micelle Extraction						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein Yield	Main findings	Reference
Defatted grape seeds powder	Reverse micelles were prepared with CTAB (20–40 mM), the aqueous solution was crude protein solution with added NaCl to adjust the ionic strength. Forward extraction: Optimization of the system applying a box-Behnken design.	–	–	Forward extraction: Up to 82.3%.	Optimum forward extraction conditions were 39 mM CTAB, pH 5.6, 0.01 M NaCl and a concentration of 2.1 mg/mL of crude protein.	Zhang et al. (2017)
Soybean flour	Reverse micelles system was 0.05 M AOT in hexane, $W_0 = 18$ adjusted by the addition of 0.05 M of KCl at pH 7.5. Forward extraction: Soybean flour and reverse micelles ratio 1:20 (w/v), mixed for 30 min at 45 °C. Backward extraction: Ratio 1:1 (v/v) between the forward extracted solution and 1 M KCl phosphate buffer (50 mM; pH 7.0). The mixture solution was stirred for 60 min at 60 °C.	37.53%	Aqueous extraction: 80.6% AOT reverse micelle: 83.6%	Aqueous extraction: 61.53% AOT reverse micelle: 72.40%	AOT reverse micelles extraction improved the hardness, adhesiveness, gumminess and chewiness of the resulting soybean protein ingredients	Zhao et al. (2015b)
	Forward extraction: Soybean protein (1 mg/mL); ratio	36.92%	–	Forward extraction:	Forward extraction optimum conditions:	

Defatted soybean flour	between the aqueous and organic phases of 1:1 (v/v). Extraction for 15 min at 25 °C. Backward extraction: The supernatant was mixed with an equal volume of stripping phase for 30 min at 25 °C followed by centrifugation.			22.3%–100% Backward extraction: Up to 100%	120 mM of AOT, pH 5.5, 0.8 M of KCl. Backward extraction: Aqueous phase (pH 5.5) resulted in 100% extraction of soybean protein from the organic phase.	Zhao et al. (2010)
Defatted peanut cake	Reverse micelles were obtained with AOT dissolved in hexane (90 mM). Different concentrations of KCl (0.00-0.25 M) and pH (6.0-8.5) were also considered. W_0 adjusted at 5-21. Forward extraction: Peanut cake flour amount (0.6–1.6 g), particle size (5–300 μm), temperature (30–55 °C), and time (20–120 min).	(N × 5.45) 46.63%	–	Forward extraction: 88.12%	Optimal forward extraction efficiency was reached at pH 7.0, water content of 18, 40 °C, for 40 min and 0.05 M of KCl.	Zhao et al. (2015a)
Soybean flour	Reverse micelles: AOT in hexane and water (0.05 M AOT, $W_0 = 18$, and a ratio of 1:20 between the soybean flour and the reverse micelle phase). Forward extraction for 30 min at 45 °C. Backward extraction: Mix between organic phase and forward solution extraction 1:1 (v/v), KCl (1 M), pH 7.5, for 60 min at 40 °C.	38.72%	–	–	Nitrogen solubility index, oil absorption capacity, foaming capacity, foaming stability, emulsifying capacity and emulsifying stability were found to be higher in the soybean proteins obtained through revers micelles extraction than in proteins obtained through alkaline extraction coupled to isoelectric precipitation	Zhao et al. (2018)

(continued)

Table 10.3 (continued)

Reverse Micelle Extraction						
Protein source	Extraction procedure	Protein content of starting material	Protein content of protein-enriched fraction	Protein Yield	Main findings	Reference
Defatted wheat germ flour	Reverse micelles: AOT in isooctane (3:50, w/v) and KCl solution (0.1 M), adjusted to pH 8.0 and $W_0 = 25$, at room temperature. Forward extraction with ultrasound-assisted conditions were 250–450 W of power, 10–30 min, and 20 kHz.	31.48%	–	Forward extraction: Up to 57%.	A global extraction yield of up to 45.6% was obtained which was significantly higher that the yield observed for alkaline extraction coupled to isoelectric precipitation (24.0–37.0%).	Zhu et al. (2009)
Defatted wheat germ flour	Reverse micelles: AOT in isooctane (3:50, w/v), $W_0 = 25$. Forward extraction: Flour and reverse micelle solution ratio 1:30 (w/v), for 60 min at 36 °C. Backward extraction: The isooctane was recovered and a KCl solution (0.61 M; pH 9.47) was added and was stirred for 60 min. A ternary liquid (acetone, deionized water and isooctane, 15:5:1 v/v/v) was then added to precipitate the proteins.	–	Reverse micelle extraction: 98.38% Alkaline extraction and isoelectric precipitation: 81.63%	Reverse micelle: 30% Alkaline extraction and isoelectric precipitation: 28%	Proteins extracted by reverse micelle extraction had a higher nitrogen solubility index, fat absorption capacity, foaming capacity and stability, and emulsifying stability, compared to alkaline extracted and isoelectrically precipited proteins.	Zhu et al. (2010)

AOT Sodium bis (2-ethylhexyl) sulphosuccinate; *CTAB* ceryl-trimethyl-ammonium bromide; W_0 water content

backward extraction protocol for the extraction of protein from defatted wheat germ in RM system. The initial total protein of wheat germ was 31.48%. The RM were obtained by solubilizing AOT in isooctane at a ratio of 3:50 w/v, W_0 adjusted to 25 by 0.1 mol/L KCl solution, with a pH of 8, for 60 min at 36 °C. For the backward extraction, 10 ml of the supernatant was distilled and the isooctane recovered.

A KCl solution was added (volume ranging between 0 and 2.5 mL, pH 7 and 10, and concentration ranging between 0.25 and 1.25 mol/L) to the resulting solution. The resulting precipitate was washed with 65% ethanol to remove any remaining surfactant, and the protein precipitate was vacuum dried at 30 °C. Optimum conditions for the backward extraction were identified as follows: KCl concentration of 0.61 mol/L, a pH of 9.47, and a volume of 1.0 mL. The optimum conditions enabled to increase the backward extraction efficiency up to 80%; these changes also saved water and offered the possibility of obtaining a nearly pure precipitate. Wang et al. (2021) also studied the optimum parameters to improve backward extraction of walnut protein in RM. For forward extraction, the defatted walnut flour (46.96% protein) was mixed with AOT/hexane in a ratio of 1:50 w/w, during 50 min at 40 °C, and then centrifuged. The resulting clear organic phase containing the protein was used for backward extraction. For the backward extraction, the organic phase containing the walnut protein was added to a 1 M KCl solution followed by microwave extraction where three independent variables were assessed: extraction time (25, 30, and 35 min), extraction temperature (40, 45, and 50 °C), and KCl solution—forward extraction solution ratio (1:1, 2:1, and 3:1). Then the two phases were separated by centrifuging at 4000 *g* for 10 min. The optimal conditions were found to be 30 min, 45 °C, and an aqueous solution to forward extraction solution ratio of 3:1 (v/v) which resulted in a backward extraction yield of 95.43 ± 2.08%. Moreover, the extracted protein showed excellent solubility, foaming, water holding and oil absorption capacity.

The study of Zhang et al. (2017) aimed at optimizing the forward extraction of proteins from grape seeds by RM. Response surface methodology was used to optimize the extraction conditions by implementing a Box-Behnken experimental design. First, the grape seeds were milled and sieved; the resulting powder was defatted with *n*-hexane for 10 h (Soxhlet extraction). In order to increase the extractability, the grape seeds defatted flour was soaked in citric acid-sodium hydrogen phosphate buffer solution (0.2 mol/L) for 1 h at a pH of 6.0. For the RM systems, Ceryl-trimethyl-ammonium bromide (CTAB) was used as a surfactant, the aqueous solution was crude protein solution after centrifugation (1500 *g* for 5 min), with added sodium chloride to adjust the ionic strength. The authors concluded that the optimum condition for forward extraction was 39 mmol/L CTAB, pH 5.6, 0.01 mol/L NaCl and a concentration of 2.1 mg/mL of crude protein. Results showed an extraction yield of 82.3% and demonstrated the potential benefits for industrial application.

Chen et al. (2014) studied the RM extraction of soybean protein with AOT in hexane (buffer solution) under different conditions, in order to get the optimum conditions in both phases (forward and backward extractions). Soybean flour that

was used as the starting material had a total protein content of 37.53%. Stock solution of varying amounts of AOT (90, 135, 180, 225, 270, 315 mmol/L) was obtained first by solubilizing AOT in hexane. A KCl solution was added at different concentrations (0.00–0.35 mol/L), pH (5.5–9.0), and alcohol amounts (0.1–0.8 vol %). For forward extraction optimum conditions were AOT concentration of 180 mmol/L, aqueous pH of 7, 0.05 mol/L KCl and 0.5% (v/v) alcohol. Under these conditions, the forward extraction efficiency of soybean protein was 70.1%. In backward extraction, the liquid phase from the forward extraction containing the soybean proteins was added to an equivalent volume of aqueous phase containing a fixed AOT concentration, KCl concentration and pH value as well as alcohol (0.1–0.8%, v/v). The backward extraction was conducted for 60 min at 35 °C. Around 92% of protein recovery was obtained after backward extraction (Chen et al. 2014).

Bu et al. (2014) evaluated two RM extraction systems, assisted by ultrasound exposure, to extract protein from soybean flour (42.2% protein; 24.7% oil): 1) AOT in isooctane and injection of KCl phosphate buffer solution to obtain a 0.08 g/mL AOT RM system; 2) AOT and Tween 85 (mass ratio 4:1) in isooctane and n-octyl alcohol (volume ratio 4:1). Backward extraction was carried out by stirring for 1 h at 30 °C the upper oil phase with a NaCl solution (0.1 M in 70% ethanol solution, pH 4) at a ratio of 2.5:1 (v/v). Results showed that AOT/Tween 85 RM extracted more protein (29.0%), than AOT RM (28.0%). However, the proteins extracted by AOT RM had a higher protein content (80.2%) than the ones extracted by AOT/Tween 85 RM (79.1%). The authors concluded that AOT/Tween 85 RM system is more efficient for forward extraction than AOT RM. Zhao et al. (2010) studied the different factors that can affect the extraction of soybean protein by RM systems. A defatted soybean flour with a protein content of 36.92%, was used for RM extraction and was dissolved in hexane, and KCl solution. Forward extraction conditions were soybean protein concentration of 1 mg/mL while the ratio between the aqueous and organic phases was 1:1 (v/v). The extraction was carried out for 15 min at 25 °C. The liquid supernatant was used for backward phase extraction and was mixed with an equal volume of stripping phase (buffer of known pH and KCl concentration) for 30 min at 25 °C. Both phases were collected after centrifugation. The forward extraction yield varied between 27% and 100%, while the backward extraction yield was up to 100% with the aqueous phase at pH 5.5. The optimum conditions were 120 mmol/L of AOT, pH value of 5.5, and 0.8 mol/L KCl.

Another study on soybean protein extraction using RM with AOT (in hexane and KCl solution) was carried out by Zhao et al. (2015b), who studied the physicochemical properties (hardness, adhesiveness, gumminess, chewiness, cohesiveness, and springiness) of proteins after RM extraction. The protein content of the soybean flour was 37.53%. The RM was obtained with 0.05 M of AOT in hexane. The water content (W_0) was adjusted to 18 by the addition of 0.05 M of KCl at pH 7.5. For forward extraction, the ratio flour/solution was 1:20 (w/v) and the mixed solution was stirred for 30 min at 45 °C before centrifugation to recuperate the supernatant solution containing the proteins. The backward extraction conditions were a ratio of 1:1 (v/v) between the forward extracted solution and 1 M KCl phosphate buffer

(50 mM; pH 7.0). The mixture solution was stirred for 60 min at 60 °C. The extraction yield of proteins in the aqueous phase was 61.53%, and 72.40% in AOT RM, while the protein content was 80.6% and 83.6%, respectively. The hardness, adhesiveness, gumminess, and chewiness of soy proteins recovered by aqueous buffer extraction were decreased by 76.08%, 67.71%, 46.49%, and 66.70%, respectively, even though the cohesiveness was increased by 35.0% compared with soy protein from RM extraction. Zhao et al. (2018) also evaluated the extracted soybean protein obtained by RMs and compared the protein extracts obtained by AOT, and alkaline extraction coupled to isoelectric precipitation. The initial protein content of soybean flour was 38.72%. RMs were made with AOT in hexane and water, the conditions were the following: 0.05 M of AOT, $W_0 = 18$, and a ratio of 1:20 between the soybean flour and the RM phase. The forward extraction was carried out for 30 min at 45 °C. The backward extraction was carried out at 40 °C for 60 min by mixing the organic phase of the forward extraction with an equal volume of 1 M KCl phosphate buffer (50 mM) and pH 7.5. The reaction mixture was centrifuged at 3700 *g* at room temperature for 10 min. The stripped aqueous phase was dialyzed at 4 °C for 24 h to purify the proteins. The nitrogen solubility index of proteins extracted by RM extraction was higher (96.9%) compared to one of proteins extracted with alkaline extraction coupled to isoelectric precipitation (88.8%). The results also showed that oil absorption capacity, foaming capacity, foaming stability, emulsifying capacity, and emulsifying stability were found to be higher in the soybean proteins obtained through RMs (those values were 2.57 g/g, 131.65%, 84.33%, 81.71%, and 82.26%, respectively) than in proteins obtained through alkaline extraction coupled to isoelectric precipitation (those values were 2.06 g/g, 112.32%, 57.15%, 50.94%, and 51.22%, respectively), while water holding capacity decreased by 8.82%. In addition, AOT RM increased the content of total amino acids, essential amino acids content, the amino acids score, and the biological value by 3.19%, 1.55%, 7%, and 6.81%, respectively, compared to soybean proteins obtained by alkaline extraction-isoelectric precipitation. These results showed that the AOT RM extraction improved the functional and nutritional properties of the protein extract while the level of off-flavor volatiles was reduced. The study by Zhu et al. (2009) showed the optimization of wheat germ protein extraction by RMs combined with ultrasound. The RMs were formed with AOT in isooctane (3:50, w/v) and KCl solution (0.1 mol/L), adjusted to pH 8.0 and $W_0 = 25$. Forward extraction with ultrasound-assisted conditions were 250–450 W of power, 10–30 min, and 20 kHz. The initial protein content of the wheat germ sample was 31.48% on a wet basis. After ultrasound extraction, the yield of extraction increased from 37% up to 57%. The backward extraction efficiency was 80% for a global RM extraction yield of up to 45.6% which was significantly higher than the yield observed for alkaline extraction coupled to isoelectric precipitation (24.0–37.0%). Zhu et al. (2010) also evaluated the extraction of wheat germ protein by RM against alkaline extraction coupled with isoelectric precipitation. RMs were obtained with AOT in isooctane in a ratio of 3:50 (w/v), the water content was adjusted to 25 with KCl solution (0.1 M; pH 8.0). Then the forward extraction was performed by adding defatted wheat germ flour to the RM solution at a ratio of 1:30 (w/v). The clear

supernatant obtained after centrifugation of the forward extraction liquid was used for backward extraction. The isooctane was recovered and a KCl solution (0.61 M; pH 9,47) was added and stirred for 60 min. To recuperate the proteins, a ternary liquid (acetone, deionized water, and isooctane, 15:5:1 v/v/v) was added, and the precipitate was then washed with 65% of ethanol. Results showed that RM with AOT had a higher extraction efficiency (30%) compared with alkaline extraction coupled to isoelectric precipitation (28%). In addition, the protein content was also higher in RM-extracted proteins (98.38 vs. 81.63%).

Zhao et al. (2015a) studied the extraction of peanut protein by RM and the optimum conditions for forward extraction. The peanut flour was defatted and analyzed for protein content by Kjeldahl (N × 5.45), presenting a total protein content of 46.63%. RMs were obtained with AOT dissolved in hexane at different concentrations (90–315 mM). Different concentrations of KCl (0.00–0.25 M) and pH (6.0–8.5) were also considered. The water content of the RM was adjusted at 5, 10, 15, 17, 19, and 21 by adding the KCl solution to the AOT/hexane mixture. In order, to improve forward extraction different parameters were studied such as peanut cake flour amount (0.6–1.6 g), particle size (5–300 μm), temperature (30–55 °C), and time (20–120 min). Optimal forward extraction efficiency was reached at 88.12% at pH 7.0, water content of 18, 40 °C, for 40 min and 0.05 M of KCl. The authors noticed that increasing the pH of extraction significantly decreased the extraction efficiency.

10.4 Conclusion and Future Perspectives

As aforementioned, the interest in plant protein sources to substitute and/or reduce animal protein consumption is on the rise. This is reflected by the large number of plant protein ingredients (protein rich flours; concentrates and isolates) that can be found on the market. Nowadays, most plant protein concentrates and isolates are produced by the alkaline extraction-isoelectric precipitation process. However, this process may affect the proteins functional properties and present some challenges from an environmental point of view due to the presence of residual proteins in the effluents following the acidic precipitation step. Some alternative technologies are available for the production of plant protein concentrates and isolates. Among these technologies, micellar precipitation and RM extraction have shown promising potential for plant protein extraction as illustrated in this chapter. Proteins isolated by micellar precipitation are less denaturated as compared to proteins isolated by isoelectric precipitation. RM extraction can be considered as environmentally friendly when biosurfactants are used. The reusability of RM enables the technology to be applied economically at industrial scale.

However, the number of studies on the application of both technologies at the industrial scale is still limited, and more research works are needed to assess their full potential for plant protein extraction. There is also a need to optimize the application of each process for the extraction of the main plant protein sources such as oilseeds, pulses and cereals, and to compare their performances with those of other processes

including the conventional alkaline extraction-isoelectric precipitation process. More studies are also needed to fully assess the impact of micellar precipitation and RM extraction on the functional and bioactive properties and on the protein quality of the extracted proteins.

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11 Application of Ultrasound Technology in Plant-Based Proteins: Improving Extraction, Physicochemical, Functional, and Nutritional Properties

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Abstract

Protein plays numerous nutritional and physiological roles in the body that are essential for life. The demands for animal proteins are estimated to double by 2050 due to the rapid growth of the world population. Plant proteins are considered a sustainable option for meeting the increasing demand for food proteins. Ultrasound technology has been applied to facilitate the extraction of proteins from plant sources, especially for the enhancement of yield and protein modifications. This chapter discusses the principles of ultrasound-assisted

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extraction (UAE) of plant proteins as well as the optimization of ultrasound-assisted protein extraction. Other topics covered include the impact of ultrasonication on the physicochemical (molecular weight, particle size, zeta potential, sulfhydryl and disulfide groups, surface hydrophobicity, and intrinsic fluorescence), functional (water solubility, foaming property, foaming stability, emulsifying property, emulsifying stability, gelling property, water and oil holding capacities), nutritional (amino acid composition, protein digestibility and anti-nutrition factor), and structural properties (primary, secondary, tertiary micro- and nano-structures) of plant proteins.

Keywords

Ultrasonication · Ultrasound · Ultrasound-assisted extraction · Plant proteins · Protein isolation · Protein digestibility · Techno-functional properties

11.1 Introduction

Protein is one of the major macronutrients and structural components of many foods, such as meat, cereals, and dairy products (Loveday 2019). Protein plays numerous nutritional and physiological roles in the body that are essential for life (Pojić et al. 2018). In the human body, proteins are one of the indispensable components of many tissues and organs, such as skin, brain, heart, liver, and kidneys. Moreover, proteins and their building units (amino acids) are used to synthesize essential physiological components, such as enzymes, hormones, lean tissue, immune function proteins, muscle mass, and bone matrix (Henley et al. 2010). Dietary protein deficiency is an important cause of malnutrition and health-related diseases, such as osteoporosis and loss of lean tissue and muscle mass, particularly in developing countries (Bourrin et al. 2000). Thus, daily protein intake data, such as estimated average requirements (EARs, g/kg/d) and recommended dietary allowances (RDAs, g/d), at different life stages have been recommended (Loveday 2019). On the other hand, proteins are commonly utilized as functional ingredients in the food industry to modify the appearance, texture, and stability of food products based on the protein properties (e.g., solubility, viscosity, foaming and foaming stability, emulsifying and emulsifying stability, gelling, water and fat absorption capacities) (Pojić et al. 2018).

Proteins for human consumption are derived from a wide variety of sources that can be generally classified as animal and plant origins. Overproduction of animal proteins for dietary purposes is not sustainable and causes negative impacts on the environment. Moreover, the demands for animal proteins, including meat, dairy, and fish products, are estimated to double by 2050 due to the rapid growth of the world population (9 billion by 2050) (Food and States 2013; Pojić et al. 2018). The replacement of animal proteins with plant proteins is considered a sustainable option for meeting the increasing consumer demand for food proteins.

Many investigations had been carried out on the extraction of proteins from plant sources for product development and dietary purposes (Pojić et al. 2018; Sun et al.

2020a). The most widely used approach to extracting proteins from food sources is the alkaline extraction and isoelectric precipitation (AEIP) method, which was established based on the isoelectric point (pI) of proteins (Zhu et al. 2006). Briefly, proteins are first solubilized in alkaline solution and subsequently precipitated at an acidic pH close to the pI (Zhu et al. 2006). However, this method has some drawbacks, such as high consumption of acid and alkali solutions, and induction of protein denaturation and exclusion of proteins with different pI (Sun et al. 2008). As such, reverse micelles (RMs) have shown promising potential to be an alternative extraction method of plant proteins (Sun and Bandara 2019). RMs are nanometer-sized aggregates of surfactant molecules within a bulk nonpolar solvent. The encapsulated water molecules function as the inner cores of RMs (Sun and Bandara 2019). Proteins are first solubilized into the aqueous inner cores of RMs and then solubilized proteins are recovered from the RM solution to obtain the protein isolates (Sun et al. 2008).

Ultrasound technique has been applied to assist both AEIP and RM methods to enhance the protein yield (Rahman and Lamsal 2021; Zhu et al. 2009). Ultrasound is a sound wave above the threshold of human auditory perception with a frequency in the range of 20 kHz to 1 GHz (Gençdağ et al. 2021). Two types of ultrasound equipment are commonly used to extract proteins, including the ultrasonic bath and probe-based systems (Tiwari 2015). The major advantage of ultrasonic bath is that the transducer does not directly make contact with the samples, but it results in a significant loss of acoustic energy. Due to the direct contact with samples and extraction solvents, probe-based ultrasound is favorable to increase protein yield and minimize acoustic energy losses (Tiwari 2015).

As a promising technique to enhance the extraction of proteins from plant sources, ultrasound offers many advantages, such as fast energy transfer, high extraction efficiency, short processing time, low unit cost, and environment friendliness and clean extraction (Gençdağ et al. 2021; Pojić et al. 2018). In addition, ultrasound-assisted extraction (UAE) has a subsequent impact on the physicochemical, functional, and structural properties of extracted proteins (Rahman and Lamsal 2021). Besides simultaneous protein extraction and modification, ultrasound technology can be directly applied to protein isolates or concentrates for the purpose of protein modification. Therefore, this chapter provides an overview of the UAE principles of plant-based proteins, optimization of ultrasound-assisted protein extraction, and impact of ultrasonication on the physicochemical, functional, nutritional, and structural properties of plant proteins.

11.2 Principles of Ultrasound-Assisted Extraction (UAE) of Plant Proteins

Ultrasound has been widely classified as low-intensity ultrasound and high-intensity ultrasound, which are characterized as high frequency (>100 kHz) and low power (<1 W/cm^2), and low frequency (20–100 kHz) and high power (>1 W/cm^2), respectively (Gençdağ et al. 2021). High-intensity (low-frequency) ultrasound is

commonly used in food extraction and processing mainly due to acoustic cavitation (Tiwari 2015). The localized pressure changes occurring in short times (a few microseconds) induce the rapid formation and collapse of bubbles in a liquid medium which is known as cavitation (Tiwari 2015). In localized zones, the ultrasonic cavitation could generate intense hydrodynamic shear forces, turbulence, high temperature, and pressure in the range of 2000–5000 K and 300–1200 bar, respectively (Rahman and Lamsal 2021). These mixing effects resulting from ultrasonic cavitation contribute to the improvement of the extraction kinetics and yield of proteins from plant foods (Lin et al. 2020; Rahman and Lamsal 2021). First, cavitation leads to disruption of plant cells, pitting of the cell membranes, and reduction of particle sizes of the cells. Scanning electron microscopy (SEM) is commonly used to visualize such disruptions of the cell walls. For example, the microstructures of *Eurycoma longifolia* powder were imaged before and after ultrasonication using SEM as shown in Fig. 11.1 (Elhag et al. 2019). The surface

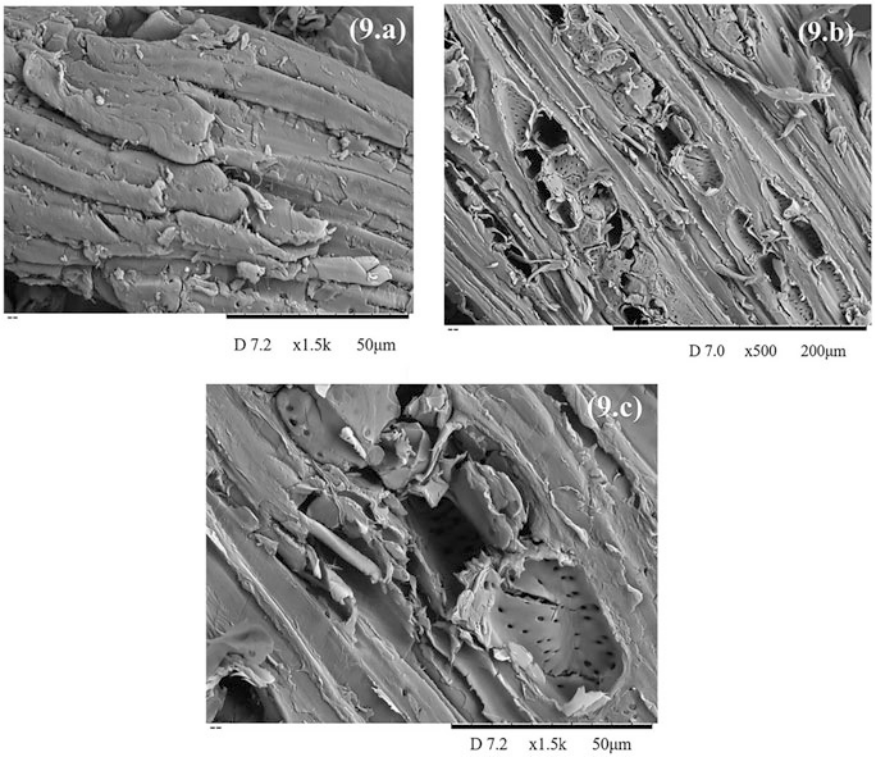

Fig. 11.1 Scanning electron microscopy images of *Eurycoma longifolia* roots (pulverized), (**a**) Dry root before ultrasonic treatment (×1500), (**b**) Root after ultrasonic treatment (×500), and (**c**) Root after ultrasonic treatment (×1500). Reprinted from Elhag et al. (2019). Optimization of protein yields by ultrasound-assisted extraction from Eurycoma longifolia roots and effect of agitation speed. *Journal of King Saud University-Science*, *31*(4), 913–930 with permission from Elsevier

of the untreated powder was smooth (Fig. 11.1a) while obvious breakages and pitting of cell membranes were observed in the samples treated with ultrasonication (Fig. 11.1b and c). Also, ultrasound enhances solvent diffusion through cell walls, thus increasing hydration and swelling of cells, and amplifying mass transfer of proteins into the solvent (Gençdağ et al. 2021; Rahman and Lamsal 2021; Tiwari 2015).

At the same time, the cavitation effects can induce physical and chemical changes in proteins; thus, ultrasound is considered an effective technique for protein modifications for food product development (Lin et al. 2020). The modifications mainly include changes in molecular interactions, structures, conformations, physicochemical properties, and functionalities of proteins. For example, cavitation breaks down water molecules to produce highly reactive free radicals such as $H^{\cdot}$ and $OH^{\cdot}$. These free radicals can react with or oxidize proteins, resulting in the disruption of interactions between protein molecules, such as hydrophobic interactions, hydrogen bonding, and disulfide bonds. As such, ultrasound can induce protein crosslinking, unfolding and aggregation, change conformations and spatial structures of proteins and alter the physicochemical and functional properties of proteins (Lin et al. 2020; Rahman and Lamsal 2021; Wen et al. 2019).

Ultrasound extraction devices include laboratory-scale, pilot-scale, and industrial-scale equipment. However, industrial-scale ultrasound extraction devices are mainly used for the extraction of bioactive compounds in the food industry. Extraction and modification of plant proteins are mainly conducted using laboratory-scale ultrasound, especially the batch type. Pilot-scale ultrasound is a commonly continuous type, but its application in plant protein extraction is limited (Preece et al. 2017; Rahman and Lamsal 2021). Therefore, future studies are needed on the application of pilot-scale and industrial-scale ultrasound in plant protein extraction.

11.3 Optimization of Ultrasound-Assisted Protein Extraction

Probe-based ultrasound is commonly used for protein extraction (Dabbour et al. 2018). Parameters that influence the protein extraction yield when ultrasound-assisted extraction (UAE) is applied include the operating frequency, ultrasonic power, ultrasonic amplitude, processing time, temperature, pH, and sample/solvent ratio (Tiwari 2015). In order to reduce cost and enhance sustainability, substantial research efforts have been made to optimize ultrasound-assisted protein extraction to maximize extraction efficiency using response surface methodology.

11.3.1 Optimization of Ultrasound-Assisted Alkaline Extraction and Isoelectric Precipitation (AEIP) Method of Protein Extraction

When ultrasonic frequency is at the level of 20 kHz, other parameters such as solvent/sample ratio, temperature, processing time, pH, ultrasonic power, and duty

cycle have been optimized. For example, at the following optimum conditions: solvent/sample ratio 18.4 mL/g, pH 9, temperature 30.8 °C and processing time 35.5 min, the maximum protein yield of 26.4% was achieved for protein extraction from evening primrose seed cake. In contrast, the lowest protein yield of 14.1% was obtained at solvent/sample ratio of 10 mL/g, pH 10, 40 °C and 15 min processing time (Hadidi et al. 2021). Likewise, UAE increased the protein extraction yield of brewer's spent grain from 45.71% to 86.16% at the optimum processing conditions (250 W, 20 min, duty cycle of 60% and 110 mM NaOH solution) (Li et al. 2021a). The extraction yield of walnut meal protein showed a rising trend when the frequency was increased from 20 kHz to 40 kHz (20, 28, 35, 40 kHz), although there was no significant difference ($P > 0.05$) between extraction efficiencies at 20 and 52 kHz (Golly et al. 2020). Moreover, dual-frequency ultrasound, combining 20/40 kHz/kHz, achieved the highest protein extraction yield (67.59%) of walnut meal protein compared to single (40 kHz, 65.69%) and triple-frequency ultrasound (20/28/52 kHz/kHz/kHz, 58.99%) (Golly et al. 2020). On the other hand, pilot-scale and lab-scale ultrasound systems resulted in different protein extraction yields of okara (Preece et al. 2017). The working conditions of lab-scale and pilot-scale systems were 400 W, 20 kHz, 100 mL plastic beaker, 13 mm radius probe tip, and 2000 W, 20 kHz, 800 mL flow cell, 38 mm radius probe tip, respectively. Although pilot-scale ultrasound significantly increased the protein extraction yield of okara by 4.2% ($P < 0.05$), the lab-scale system resulted in a higher yield of 40% after 15 min processing due to the higher energy intensity (Preece et al. 2017).

11.3.2 Optimization of Ultrasound-Assisted Reverse Micelles (RMs) Method of Protein Extraction

An ultrasound-assisted approach has been developed for protein extraction from defatted wheat germ using RMs. UAE parameters, including ultrasonic output power, processing time and pulse mode, have been optimized by response surface methodology using a three-level, three-variable Box-Behnken experimental design. The optimum extraction conditions included 363 W of ultrasonic output power, 24 min of processing time, and 2.4 s on and 2 s off of pulse mode, which resulted in the enhancement of protein yields from 37% to 57% (Zhu et al. 2009). However, limited research has been conducted to optimize the UAE parameters for enhancing the extraction efficiency of proteins isolated by RMs method. This gap is probably due to the presence of organic solvents in RMs.

11.4 Impact of Ultrasonication on the Physicochemical Properties of Plant Proteins

As shown in Table 11.1, ultrasonication affects the physicochemical properties of plant proteins, including molecular weight, particle size, zeta potential, SH and SS groups, surface hydrophobicity, and intrinsic fluorescence. Details of the ultrasonication-induced effects on the protein properties are discussed in this section.

Table 11.1 Impact of ultrasonication on the physicochemical properties of plant proteins

Physicochemical property	Type of processing	Major impact	Protein or source	Possible mechanism	Parameter responsible for effect	Reference
Molecular weight	UAE[a]	Decrease	Arachin	Dissociation of protein aggregates induced by the cavitation effects	High temperature (e.g., > 25 °C)	Sun et al. (2020b)
	UAE	Increase	Conarachin	Protein aggregation	High ultrasound energy density (e.g., 6.33 W/cm^3); long processing time (e.g., 40 min)	Sun et al. (2020b)
	UAE	No change	Proteins from soybean okara byproduct	Not mentioned	Not mentioned	Aiello et al. (2021)
Particle size	UAE	Decrease	Proteins from sunflower meal, defatted soy flakes, and pea powder	Dissociation of large insoluble protein aggregates into small particles through disrupting non-covalent interactions	High amplitude (e.g., 70%) and short ultrasonic time (e.g., 15 min)	Dabbour et al. (2018); Karki et al. (2010); Wang et al. (2020b)
	Direct application[b]		Protein isolates from *Chenopodium album*, soybean, and faba bean			Mir et al. (2019a); Wang et al., (2020b); Martínez-Velasco et al. (2018)
Zeta potential	Direct application	Decrease	Protein isolates from faba bean and chickpea	Negatively charged groups were neutralized by the exposure of hydrophobic groups or positively charged groups	Prolonged processing time (e.g., from 10 min to 20 min)	Martínez-Velasco et al. (2018); Wang et al., (2020b)
Sulfhydryl (SH) group	UAE	Decrease	Proteins from soybean okara byproduct and brewer's spent grain	Generation of hydroxyl radicals and hydrogen atoms to oxidize the free SH groups	Increased ultrasonic temperature (e.g., 60 °C and 80 °C)	Aiello et al. (2021); Li et al. (2021a)

(continued)

Table 11.1 (continued)

Physicochemical property	Type of processing	Major impact	Protein or source	Possible mechanism	Parameter responsible for effect	Reference
	UAE	Increase	Proteins from rice and Antarctic krill	Reduction in disulfide (SS) content	Not mentioned	Li et al. (2021b); Yang et al. (2018)
Surface hydrophobicity	Direct application	Increase	Pea protein isolates	Unfolding of protein molecules	Ultrasonic amplitude	Xiong et al. (2018)
	UAE		Proteins from rice	Disruption of starch–protein interaction and unfolding of protein structure	Not mentioned	Yang et al. (2018);
	Direct application	Decrease	Protein isolates from *Chenopodium album*	Protein aggregation and protection of its hydrophobic groups from the polar environment	Increased ultrasonic time (e.g., from 25 min to 35 min)	Mir et al. (2019a)

[a]Ultrasound-assisted extraction (UAE)
[b]Direct application of ultrasonication in protein isolates

11.4.1 Molecular Weight Distribution

Molecular weight (Mw) distribution is one of the important characteristics of protein isolates. The changes in the Mw distribution of proteins are commonly analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography methods (Mir et al. 2019a; Sun et al. 2020b). UAE had distinct effects on the Mw distribution of different proteins (Sun et al. 2020b; Aiello et al. 2021). For example, UAE led to the reduction of Mw of arachin, which may be due to the dissociation of protein aggregates induced by the cavitation effects, high shear stress, micro-streaming, and turbulent forces of ultrasonication process (Sun et al. 2020b). However, the percentage of high Mw conarachin (>93.19 kDa) in UAE-treated samples at different conditions were higher than those of the controls; this effect was attributed to protein aggregation induced by ultrasonication (Sun et al. 2020b). Moreover, UAE did not influence the Mw distribution of proteins from soybean okara byproduct based on SDS-PAGE under reducing conditions, but the possible mechanisms were not revealed (Aiello et al. 2021).

UAE parameters play an important role in the Mw distributions of proteins. For instance, increasing the ultrasound energy density from 1.58 to 6.33 W/cm^3 slightly increased the percentage of conarachin with high Mw (>93.19 kDa) from 19.14% to 20.30%. Furthermore, extending the duration of ultrasound treatment from 10 to 40 min significantly increased the proportion of conarachin with high Mw from 21.31% to 26.99%. However, increasing ultrasound temperature from 5 °C to 45 °C reduced the percentage of fraction with high Mw from 27.78% to 20.21%. Overall, longer ultrasound treatment duration and high ultrasound energy density would increase the protein Mw as a result of protein aggregation (Sun et al. 2020b).

11.4.2 Particle Size and Zeta Potential

The particle size of protein plays an important role in the functional properties, such as emulsifying and foaming abilities (Wang et al. 2020a). It has been demonstrated that UAE decreased the particle size of extracted proteins from sunflower meal, defatted soy flakes, and pea powder (Dabbour et al. 2018; Karki et al. 2010; Wang et al. 2020b)). More research efforts have been made in investigating the impact of ultrasonication on protein isolates. Mir et al. (2019a) reported that ultrasonication significantly reduced the particle size of album protein isolates compared to the control protein; the biggest decrease (from 245.63 μm to 134.28 μm) was observed when sonication time was 25 min. Moreover, ultrasonication decreased the particle sizes of two types of soy protein isolates (denatured by heat moisture or alcohol) from 103.22 μm and 222.52 μm to 64.87 μm and 30.80 μm, respectively (Zheng et al. 2019). Reduction in the particle size of proteins occurred owing to the dissociation of large insoluble protein aggregates into smaller particles, an effect attributed to the cavitation phenomenon and micro-streaming and turbulent forces generated by ultrasound (Mir et al. 2019a; Wang et al. 2020a). Non-covalent

interactions, such as hydrogen bonds, electrostatic and hydrophobic interactions, can be disrupted during ultrasound process (Dabbour et al. 2018; Wang et al. 2020a). In addition, ultrasonic parameters, including amplitude and processing time, influenced the particle size of faba bean protein isolates. Specifically, at higher amplitude (70%) and shorter ultrasonic time (15 min), particle size of the protein isolate was smaller (225.33 nm). In contrast, larger protein particles (265.21 nm) were produced at lower amplitude (50%) and longer ultrasonic time (30 min) due to the formation of soluble protein aggregates (Martínez-Velasco et al. 2018).

Zeta potential is a physical parameter used to quantify the surface charge of proteins, which influences the extent of protein aggregation (Wang et al. 2020a). The impact of ultrasonication on plant protein isolates has been well studied, and it mostly decreased the net surface charge of proteins (Martínez-Velasco et al. 2018; Wang et al. 2020a). For example, the zeta potential of faba bean protein significantly decreased from −30.15 mV for untreated sample to −24.65 mV for UAE sample (Martínez-Velasco et al. 2018). Prolonged ultrasonic processing time (from 10 min to 20 min) further reduced the zeta potential of chickpea protein isolates from −28 mV to −24 mV (Wang et al. 2020a). The net surface charge of proteins depends on the number of hydrophobic groups, positive charged and negatively charged groups located on the surface of protein molecules. The reduction of net surface charge induced by ultrasonication is attributable to the exposure of hydrophobic groups or positively charged groups, which can neutralize the negatively charged groups on the protein surface (Martínez-Velasco et al. 2018; Wang et al. 2020a). Ultrasonication-induced protein cross-linkages may have also contributed to reducing the surface charge of the protein molecules.

11.4.3 Contents of Free Sulfhydryl (SH) and Disulfide Bond (SS)

Disulfide bond (SS) is an important chemical bond for forming and stabilizing the tertiary structure of proteins. UAE can lead to the formation or breakage of SS bond, which in turn causes the interconversions of SH and SS. Measurement of the contents of free SH and SS groups is commonly used to provide insights into changes in the protein tertiary structure (Aiello et al. 2021; Li et al. 2021a; Lian et al. 2021). Some research findings suggested that the free SH content of proteins exhibited a declining trend during UAE treatment (Aiello et al. 2021; Li et al. 2021b). In detail, in comparison with untreated soy okara protein, UAE at 20 °C markedly reduced the free SH content from 59.4 μmol/g to 7.4 μmol/g. Increase in ultrasonic temperature to 60 °C and 80 °C resulted in an additional reduction in free SH content to 5.3 μmol/g and 2.9 μmol/g, respectively (Aiello et al. 2021). Thermolysis and cavitation may generate hydroxyl radical, which is considered as the dominant factor in the oxidation of the free SH groups (Aiello et al. 2021; Li et al. 2021a). However, other studies reported contradicting results that UAE increased the content of free SH groups (Li et al. 2021b; Yang et al. 2018). For example, the SH content of rice protein isolated by UAE (86 μmol/g) was significantly increased by 14.67% compared to the control, and its SS content reduced by 26.5% (Yang et al.

2018). The discrepancy in these results might be related to differences in the ultrasound intensity, protein types, and structural properties (Li et al. 2021a, b).

11.4.4 Surface Hydrophobicity

Surface hydrophobicity (S_0) of proteins is an index of the number of hydrophobic groups that are exposed on the surface of protein molecules (Mir et al. 2019a). As such, surface hydrophobicity is commonly used to indicate the changes of protein conformations and structures. The higher the S_0 value, the higher the level of protein unfolding (Pan et al. 2020). Generally, ultrasonication causes the enhancement of surface hydrophobicity of proteins (Xiong et al. 2018; Yang et al. 2018). For example, when rice protein was extracted by UAE and α-amylase degradation methods, the surface hydrophobicity of extracted proteins was increased owing to the disruption of starch–protein interaction and unfolding of protein structure (Yang et al. 2018). Treatment of pea protein isolates by ultrasonication with a 20-kHz probe at different amplitudes (30%, 60%, 90%) for 30 min significantly enhanced the surface hydrophobicity compared to untreated proteins. The ultrasonic amplitude was positively associated with the surface hydrophobicity (Xiong et al. 2018), possibly due to ultrasonication-induced unfolding of protein molecules, which in turn enhanced the exposure of buried hydrophobic groups (Mir et al. 2019a). However, surface hydrophobicity of album protein isolates was significantly decreased when ultrasonic time was increased from 25 min to 35 min. This decrease is possibly due to an excessive ultrasonic processing resulting in protein aggregation and protection of the hydrophobic groups from the polar environment (Mir et al. 2019a).

11.5 Impact of Ultrasonication on the Functional Properties of Plant Proteins

Ultrasonication can be used in both destructive and non-destructive applications. High-intensity ultrasound waves are often used for destructive applications, such as emulsification, diffusion, and extraction, whereas low-intensity ultrasound waves are commonly used for non-destructive applications, such as analyzing the sugar content, acidity, ripeness, and firmness of food (Malik et al. 2017). The use of high-intensity ultrasound is considered a green approach to protein extraction as it has the potential to significantly lower the amount of both alkali solvent needed in protein recovery and acid solvent used in isoelectric precipitation (Malik et al. 2017). As discussed, critical factors to consider in the extraction of plant proteins include power, frequency, intensity, duration, pH, and temperature (Kumar et al. 2021). These parameters also influence the functional properties of the isolated proteins. High-intensity ultrasound waves for both protein extraction and treatment of extracted proteins generate a combined effect of shear stress, dynamic agitation, cavitation, and turbulence, which alter the structure and functional properties of food

relative to low-intensity ultrasound waves (Li et al. 2021a; Malik et al. 2017). The changes in structure and functional properties are a result of irreversible changes in molecular features. Nonetheless, both longer treatments and high-power ultrasonic waves can result in reaggregation of the exposed hydrophobic moieties linking disordered proteins to form macromolecular aggregates (Rahman and Lamsal 2021). This could cause a reversal in the trend of some of the functional properties. This section discusses the influence of UAE and ultrasonication on the functional properties of plant proteins.

11.5.1 Water Solubility

Solubility is one of the most practical ways to analyze structural changes and differences in functionality among various extracted plant proteins at a specified pH. It is a reliable index to determine the functionality of protein extracts (Hu et al. 2013). Protein solubility is a function of the extent of protein denaturation, particle size, intermolecular attraction or repulsion, and surface hydrophobicity (Hu et al. 2015; Jiang et al. 2014; Nazari et al. 2018). Hadidi et al. (2020) modified the conventional technique of alkaline extraction and isoelectric precipitation of alfalfa leaves by including ultrasonication and ultrafiltration in the extraction process. With the modified extraction technique, the solubility of proteins was slightly higher (94.7%) at pH 8.0 relative to other techniques, such as heat-coagulation extraction (92.2%) at pH 9.0 and alkaline solubilization-isoelectric precipitation extraction (93.6%) at pH 8.0. Protein solubility is generally at its lowest concentration at the isoelectric point (~ pH 4.0–5.0) where the net charge of the protein is 0, while an alkaline medium improves solubility. Similarly, Golly et al. (2020) reported an increase in protein solubility upon using single-frequency (93.0%), dual-frequency (85.8%), and triple-frequency (81.3%) ultrasonication in the extraction of protein isolates from walnut meal relative to the traditional alkaline solubilization-isoelectric precipitation (41.2%) extraction process. On another note, Aiello et al. (2021) demonstrated that protein solubility from soybean okara by-products could be enhanced by using ultrasound-assisted extraction with increasing temperature from 20 °C up to 80 °C. This approach was also useful in maintaining the consistency of the protein dispersion while increasing solubility (Aiello et al. 2021).

Jiang et al. (2014) used low frequency (20 kHz) ultrasonication at different powers (0, 150, 300, 450 W) to modify the properties of black pea protein isolate dispersions. Relative to the native proteins, there was an enhancement in the solubility of proteins treated with ultrasonic waves. Similarly, Jambrak et al. (2009) observed an improvement in the solubility of soy protein isolates and concentrates that were treated with either ultrasonic probes (20 kHz probe, 30 min, 64.3–78%) or ultrasonic bath (40 kHz bath, 15 min, 64.3–82%) systems. This is because sonication results in a breakdown of hydrogen and hydrophobic bonds due to cavitation resulting in a decrease in particle sizes of protein dispersions (Jambrak et al. 2009; Mir et al. 2019a). Smaller particle sizes provide a larger surface area, which improves electrical conductivity to enhance interaction with water molecules

(Mir et al. 2019a; Nazari et al. 2018). Additionally, the breakdown of hydrogen and hydrophobic bonds causes protein structures to unfold, which exposes more hydrophilic moieties to the polar environment (Mir et al. 2019a). This results in an increase of protein solubility. Furthermore, Hu et al. (2013) reported that ultrasonic treatment increased the protein solubility, but had no apparent effect on the protein profiles of soy protein isolates. 7S globulins (β-conglycinin subunits) of soy protein showed higher solubility than the 11S globulins (glycinin subunit). This is because the former are trimeric proteins whereas the latter are hexameric proteins. Notably, continuous application of ultrasonic waves could have a contrasting result due to the disruption of covalent bonds, which can then react with oxygen species to form protein aggregates with higher molecular weights (Rahman and Lamsal 2021). This phenomenon decreases the solubility of proteins in water. The optimum duration of sonication for each protein sample differs and must be determined to ensure optimum solubility.

11.5.2 Foaming Property and Foaming Stability

Ultrasound waves can be used to improve the foaming capacity and stabilizing activity of plant protein extracts. Foaming is formed when whipped proteins rapidly diffuse into the air–liquid interface to form a viscoelastic layer around the interface (Kamani et al. 2021). This phenomenon decreases the air–liquid surface tension and causes partial denaturation and unfolding of the protein (Singh 2011). The foaming capacity of proteins is the amount of interfacial area that is formed by whipping protein solutions to disperse air into the solution (Mauer 2003). The improvement in foaming capacity is controlled by three main processes; transportation, penetration, and reorganization of the molecules at the air/water interface (Malik et al. 2017). These processes are also influenced by physicochemical factors, such as the orientation of proteins, surface hydrophobicity, particle size, solubility of proteins, homogenization effect, decrease in intermolecular interactions between protein molecules, and structural flexibility of the surfactant (Hu et al. 2013; Malik et al. 2017; Mir et al. 2019a). Li et al. (2021a) used ultrasound-assisted extraction with operating powers between 150 W and 350 W to extract protein isolates from brewer's spent grain. There was an increase in both foaming capacity (112%, 250 W) and stability (111%, 300 W) due to the increase in protein flexibility and exposure of hydrophobic groups. Also, Golly et al. (2020) utilized a multi-frequency countercurrent ultrasonic treatment in the extraction of protein isolates from walnut meals and observed a similar trend in both foaming property and foaming stability of samples in the order: dual frequency > triple frequency > mono-frequency > alkaline-isoelectric precipitation (control). The trend observed in the foaming capacity and stability followed the surface hydrophobicity pattern. Similarly, using *Chenopodium album* seed, Mir and colleagues observed an increase in foaming capacity which was linked to an increase in surface hydrophobicity (Mir et al. 2019a). Likewise, Nazari et al. (2018) observed an increase in foaming capacity from 271 mL to 749 mL and stability from 4.37 min to 95.70 min when the amplitude of sonication was increased to 73.95 W/

cm^2. The increase in the amplitude of sonication caused a change in protein structure, which exposed more hydrophilic moieties. Xiong et al. (2018) reported that pea protein isolates treated with ultrasonic waves (90%, 30 min) had a 1.38-fold higher foaming capacity than the untreated pea protein isolates. Notably, foaming properties differ with samples, duration of sonication, standing time, and sample preparation conditions used to obtain a viscoelastic film. Beyond the optimum sonication time, the size of protein-induced foam bubbles continues to increase resulting in the desorption of the ultrasonically treated protein molecules from the air–liquid interface (Li et al. 2017b). Consequently, the desorbed protein molecules easily interact using hydrophobic forces to induce protein aggregation. An increase in protein aggregation exposes more of the hydrophobic moieties to the polar environment resulting in a decrease in protein solubility, activity, and foaming properties (Kamani et al. 2021; Li et al. 2017b; Xiong et al. 2018). A similar mechanism resulting in a reduction in foaming properties is observed when the optimum foam standing time is exceeded.

11.5.3 Emulsifying Activity and Emulsifying Stability

Emulsifying activity and stability are used to characterize the emulsifying properties of protein extracts for diverse food applications. Emulsifying activity is generally described as the potential for proteins to adhere to the interface of oil and water whereas emulsifying stability is the ability of proteins to remain at the oil–water interface following emulsion storage or heating. During protein emulsification, interfacial tension is reduced as a result of the proteins having their hydrophobic moieties realigning with the oil phase whereas the hydrophilic moieties align with the water phase (Kamani et al. 2021). Application of ultrasound waves to protein extracts can enhance their emulsifying activity and stability by means of denaturation to enhance molecular flexibility, poly-dispersibility, solubility, and surface hydrophobicity (Mir et al. 2019b; Wu et al. 2020). Additionally, Kamani et al. (2021) noted that an improvement in emulsifying activity index of ultrasonicated proteins extracted from black gram by-product was a function of an increase in negative surface charge, which enhanced the adherence of the modified proteins toward oil located at the oil–water interface. On the contrary, prolonged sonication results in the denaturing of protein structures, aggregation of denatured proteins, and reduction in oil–water interfaces (Malik et al. 2017; Mir et al. 2019b). However, there have been mixed reports on the effect of ultrasonication treatment on protein extraction and protein extracts relative to the control proteins. Whereas an increase in emulsifying stability index was observed in dephenolized sunflower meal (Malik et al. 2017), plum seed (Xue et al. 2018), quinoa seeds (Mir et al. 2019b), and amaranth seeds (Tomé Constantino and Garcia-Rojas 2020), there was an opposite effect on the treated proteins extracted from black gram by-product relative to the control proteins (Kamani et al. 2021). Golly et al. (2020) observed a decrease in the emulsifying activity index of protein isolates extracted from walnut meals with the assistance of varied levels of ultrasonication frequency, namely mono-frequency

(67.8%), triple frequency (66.9%), and dual frequency (66.8%) when compared to the control (69.4%) extracted using the traditional AEIP technique. However, the reverse order was observed in the emulsifying stability using the same techniques. On the other hand, Li et al. (2021a) reported an increase in emulsifying activity index from 33.64 to 42.32 when the power of ultrasonic-assisted extraction of brewer's spent grain was increased from 150 W to 350 W, and an increase in emulsifying stability index from 64.67 to 81.62 when ultrasonic power was increased from 150 W to 250 W. This effect was followed by a subsequent decrease in emulsifying stability index due to protein aggregation. Further studies are necessary to ascertain the influence of ultrasonication treatment on protein extracts in terms of emulsifying activity and emulsifying stability.

11.5.4 Gelling Property

Gels are unique elastic semi-solid substances with the capacity to withstand flow when subjected to pressure. Gels have the capacity to retain their structural shape to some extent due to their elasticity. The onset of gelation is characterized by an increase in storage modulus (G′) (Tang et al. 2009). G′ indicates the amount of energy inherent in the structure of the food gel. Moreover, the loss modulus (G′′) of food gels, which indicates the dissipated energy in one cyclic load, is characteristically lower than the G′. Gelation is an essential functional attribute used in the processing of certain food products such as confectionery products, jams, yogurts, desserts, meat products, and cheeses (Saha and Bhattacharya 2010). Protein gels are formed as a result of the interconnectivity between denatured protein aggregates in a liquid phase that exceeds a critical concentration (Resendiz-Vazquez et al. 2017). The formation of protein aggregates is influenced by the molecular structure of proteins. Several studies have shown that treatment of plant protein extracts with ultrasonication could induce the formation of protein gels due to the generated heat (Hu et al. 2013; Tang et al. 2009). Application of ultrasonication to the plant protein extracts could enhance the solubility of proteins, decrease the size of protein particles and create a more homogeneous particle dissolved in the liquid phase. This phenomenon results in the formation of thicker and more homogenous interconnectivity to form a gel (Hu et al. 2013). Additionally, the exposure of hydrophobic residues enhances the formation of a firmer gel network (Hu et al. 2013; Resendiz-Vazquez et al. 2017).

11.5.5 Water and Oil Holding Capacities

Protein interactions with water are expressed in many synonymous terms, such as water hydration, water holding, water adsorption, water binding, and water imbibing. The water holding capacity of proteins is the capacity to preclude the release of water from the three-dimensional structure of proteins when subjected to external influences such as pressing, grinding, centrifugation, or heating (Zayas 1997). On

the other hand, oil and fat binding capacity is the capacity of proteins to entrap oil/fat due to the bulk density and lateral nonpolar protein chains (Olivos-Lugo et al. 2010). Generally, an improvement in water holding capacity enhances the visual acceptability and texture (such as juiciness and viscosity) of food whereas oil holding capacity enhances the flavor retention of foods (Haque et al. 2016). Of note, there are mixed reports on the influence of ultrasonication on the water/oil holding capacity, which could be due to the use of different protein samples and processing conditions (Olivos-Lugo et al. 2010; Resendiz-Vazquez et al. 2017; Xue et al. 2018). Hadidi et al. (2020) used a modified conventional technique of alkaline extraction-isoelectric precipitation of alfalfa leaves by including ultrasonication and ultrafiltration in the extraction process, leading to improved results for WHC and OBC when compared with other techniques. According to Hadidi et al. (2020), their ultrasonic modified extraction technique yielded WHC and OBC of 4.35 g water/g and 4.88 g oil/g, respectively, which was significantly higher than results obtained using alkaline solubilization-isoelectric precipitation extraction (3.90 g water/g and 4.27 g oil/g) and heat-coagulation extraction technique (3.34 g water/g and 3.95 g oil/g). On the other hand, alfalfa protein isolates produced by Fiorentini and Galoppini (1981) using the conventional alkaline-isoelectric precipitation resulted in a WHC and OBC of 4.5 g water/g and 4.1 g oil/g, respectively. Of note, a negative correlation exists between the water and oil holding capacity (Resendiz-Vazquez et al. 2017; Xue et al. 2018). Ultrasonication can influence the oil binding capacity of protein extracts by denaturing proteins resulting in protein unfolding and exposure of hydrophobic groups to interact with lipids (Olivos-Lugo et al. 2010). However, to enhance the water holding capacity, ultrasonication parameters need to be optimized to ensure exposure of hydrophilic moieties to the polar environment, effective protein solubility, smaller particle sizes, and the absence of protein aggregation (Kamani et al. 2021; Xue et al. 2018).

11.6 Impact of UAE on the Nutritional Properties of Plant Proteins

One of the challenges of proteins from plant sources is their low nutritional value compared to animal proteins due to inadequate amounts of essential amino acids, presence of anti-nutritional factors, and low protein digestibility. Pre-treatment techniques such as UAE could improve the nutritional properties of plant proteins (Golly et al. 2020; Pan et al. 2020; Hadidi et al. 2020). Thus, understanding how direct ultrasound treatment and UAE changes amino acid composition, protein digestibility, and anti-nutritional factor contents are important considerations for its application in the food industry.

11.6.1 Amino Acid Composition

In general, UAE increases the total and free amino acid contents following extraction. For example, the total amino acid quantity of UAE-treated pea protein isolates (PPI) increased by 12.6% compared with PPI extracted by the alkaline method (Wang et al. 2020b). Similarly, a 2.2-time higher free amino acid content was reported following ultrasonication pre-treatment of corn steep liquor compared to the sample without ultrasonic treatment (Trakselyte-Rupsiene et al. 2021). This increase is likely due to the direct effect of UAE on protein extraction, which enhances the protein content of the resulting isolates (Wang et al. 2020b). Application of ultrasound as a pre-treatment induces molecular unfolding and protein structural changes caused by cavitation (Li et al. 2017a). Thus, loosening of the plant tissue microstructure allows for easier protein extraction, thus increasing total amino acid content in the process (Li et al. 2017a; Golly et al. 2020).

Despite the consistent increase in total amino acid content liberated following UAE, the change in amino acid profile upon UAE treatment appears to be largely dependent on the initial amino acid composition of the plant source. An overall increase in hydrophobic amino acids was consistently observed in UAE samples (Golly et al. 2020; Hadidi et al. 2021; Sun et al. 2021). However, the change in amino acid profile was not uniform across varying plant protein sources. For example, UAE of protein isolates from Walnut (*juglans regia* L.) resulted in an increase in hydrophobic residues Ala and Leu, as well as polar residues Asp, Ser, Thr, and Cys (Golly et al. 2020). Moreover, a decrease in aromatic residues Phe and Tyr, and most charged residues including Arg, His, and Glu were also noted (Golly et al. 2020). Similarly, an increased hydrophobic content of UAE pre-treated peanut and rice dreg flour protein isolates has also been reported (Sun et al. 2021; Li et al. 2017a). As mentioned previously, the application of ultrasound may have enhanced the exposure of embedded hydrophobic residues as a result of the cavitation effects which altered the molecular structure (Golly et al. 2020; Sun et al. 2021). To contrast, an increase in polar and positively charged Glu, Asp, and Arg amino acids were also noted in peanut and vine annual plant (*Dolichos lablab* L.) protein isolates (Sun et al. 2021; Zhao et al. 2021). Likewise, an increase in positively charged Lys residues along with hydrophobic Met residues was observed in UAE-treated protein from rice dreg flour (Li et al. 2017a).

11.6.2 In Vitro Protein Digestibility

Samples treated with UAE resulted in differing effects on protein digestibility. For example, no significant effect on protein digestibility was observed in rice bran, canola protein isolate, and almond milk proteins following UAE treatment compared to conventional extraction methods (Bedin et al. 2020; Vanga et al. 2020a; Flores-Jiménez et al. 2019). Furthermore, the relative protein digestibility of faba beans following UAE treatment slightly decreased from 68.42% to 65.98% (Martínez-Velasco et al. 2018). In contrast, direct ultrasound treatment was reported to be an

effective method for enhancing the enzymatic hydrolysis of wheat germ proteins (Jia et al. 2010) and buckwheat proteins (Jin et al. 2021). UAE treatment significantly increased in vitro protein digestibility of soy protein by 12.4%, and soymilk protein by 84.03% (Khatkar et al. 2020; Vanga et al. 2020b). Similarly, in vitro digestibility of rapeseed napin increased following an increase in UAE intensity from 10% to 40%; however, this effect was diminished with increasing ultrasound intensity from 40% to 70% (Pan et al. 2020).

UAE likely increases digestibility by loosening the protein via the disruption of hydrogen bonds and Van der Waals interactions. As a result, this allows for hydrophobic sequences and other regions embedded within the protein to be exposed outside, allowing for increased access of the proteases to buried cleavage sites (Jia et al. 2010; Khatkar et al. 2020). Alternatively, the promotion of random coil formation, in addition to protein denaturation, equally increases protein digestibility as observed with UAE-treated sorghum gluten-like flour protein isolates (Sullivan et al. 2018). However, increased ultrasound intensity can also result in the decrease in digestibility possibly due to the formation of S-S bonds, which enhances protein aggregation (Pan et al. 2020). Furthermore, a negative correlation between β-sheet content and digestibility has been previously established (Vanga et al. 2020b). The increase in surface hydrophobicity as a result of extended exposure to UAE treatment can enhance aggregation due to increased β-sheet content, thus reducing protein digestibility (Vanga et al. 2020b; Golly et al. 2020; Sun et al. 2021; Wang et al. 2020b). This illustrates the importance of optimization of UAE conditions, e.g., ultrasound intensity, duration, or other parameters, for the different protein sources. It is also important to note that the relative protein content as well as compactness of the molecular configuration, can also affect enzyme accessibility and protein digestibility.

11.7 Impact of UAE on the Structural Properties of Plant Proteins

11.7.1 Primary Structure (Protein Profile)

Several studies have reported little or no changes in the protein primary structure of UAE-treated samples compared to untreated control. For example, *Ganext* beans isolated via UAE exhibited the same protein profile compared to the control (Lafarga et al. 2018). Similar results were observed for rice bran protein (Ly et al. 2018) and vine annual plant *Dolichos lablab* L. protein (Zhao et al. 2021) isolated with UAE compared to their respective controls. It is likely that UAE is mild on proteins, thus limiting any noticeable protein degradation or other covalent modifications that affect the structural integrity and profile of the plant proteins (Lafarga et al. 2018; Ly et al. 2018; Zhao et al. 2021). Notably, UAE preferentially interferes with hydrogen bonding, ionic interactions, and other non-covalent interactions (Zhao et al. 2021).

Direct ultrasound treatment of protein isolates provided different results than those observed with UAE. For example, direct ultrasound treatment of album (*Chenopodium album*) seed isolates appeared to alter the molecular structure of proteins compared to the native control (Mir et al. 2019a). Following sonication, the appearance of two intense bands of smaller molecular weights suggested that prolonged exposure of ultrasonication produced cavitation forces strong enough to cleave peptide bonds of the proteins (Mir et al. 2019a). Similarly, a reduction in molecular weight following ultrasonication was observed in jackfruit (*Artocarpus heterophyllus*) seed protein isolate (Resendiz-Vazquez et al. 2017). These effects could have also resulted from oxidative cleavage of proteins induced by the reactive species generated during ultrasound treatment.

11.7.2 Secondary Structure

Ultrasound treatment affects the secondary structure of proteins. For example, ultrasound treatment of the walnut (*Juglans regia* L.) meal, pea protein, and peanut proteins resulted in the decrease of α-helix content with a concomitant increase in β-sheet, β-turn, and random coil contents compared to the conventionally extracted control (Golly et al. 2020; Sun et al. 2021; Wang et al. 2020b). However, a decrease in the β-turn content was also observed with the increase in β-sheets for some proteins (Golly et al. 2020; Sun et al. 2021; Wang et al. 2020b). Increased exposure of embedded hydrophobic residues and surface hydrophobicity caused by ultrasound treatment disrupts hydrogen bonding and reduces aggregate size in favor of β-sheet formation (Golly et al. 2020). It is suggested that brewer's spent grain subjected to UAE also resulted in an exposure time-dependent increase in β-sheet content, where an increase in α-helical and β-turn content was initially observed followed by a subsequent decrease after prolonged sonication time (Li et al. 2021a). Random coils had the opposite trend (Li et al. 2021a). The breaking of non-covalent bonds by UAE likely enhances the formation of more flexible structures, which encourages internal rearrangements that favor the adoption of β-sheet conformations and the formation of aggregates (Golly et al. 2020; Lian et al. 2021; Sun et al. 2021). Furthermore, UAE exposure time influences the changes in protein secondary structure. Sun et al. (2021) demonstrated that the secondary structure contents of peanut protein isolates were not altered during short exposure times (less than 30 min). However, increased exposure time beyond 50 min resulted in a simultaneous decrease in α-helical content and an increase in β-sheet content (Sun et al. 2021). Conversely, UAE of proteins from other sources, such as peanut flour and evening primrose (*Oenothera biennis I.*) seed cake, had no effects on the secondary structure contents (Hadidi et al. 2021; Ochoa-Rivas et al. 2017).

Given the complexity of the extraction matrix, protein structure, and intermolecular interactions, it is challenging to explain the fundamental basis of the effects of UAE on the protein secondary structure. Nonetheless, the type of protein is an important consideration. For instance, peanut protein isolate arachin subjected to UAE resulted in an increased α-helical content and decreased β-sheet and β-turn

compared to untreated control (Sun et al. 2020b). On the other hand, the high α-helical content of conarachin resulted in no further changes to the secondary structure following UAE (Sun et al. 2020b). This suggests that the type of secondary structures of the native proteins determine the extent of the effects of UAE.

11.7.3 Tertiary Structure

Given the effect on secondary structure, ultrasonication also influences the protein tertiary structure. The changes in conformations and tertiary structures of proteins are commonly indicated by the shift of the intrinsic protein fluorescence when the polarity of the environment of the tryptophan (Trp) and tyrosine (Tyr) residues varies (Pan et al. 2020; Wang et al. 2020b). This shift includes changes in the maximum fluorescence intensity and maximum fluorescence emission wavelength. Variable effects of UAE on the tertiary structure of proteins have been reported (Pan et al. 2020; Sun et al. 2020b; Wang et al. 2020b). For example, UAE induced the aggregation of pea proteins and resulted in fewer Trp residues exposed to the hydrophilic environment under longer ultrasound time (50 min) compared to control protein extracted by the alkaline method. On the contrary, shorter ultrasound time (30 min) enhanced the exposure of Trp residues to the surface due to protein unfolding (Sun et al. 2021). Furthermore, ultrasound treatment time influences the maximum fluorescence emission wavelength. UAE caused a red shift for conarachin, possibly resulting from the efficiency of energy transfer between Tyr and Trp, indicating the higher exposure of the chromophores to solvent. However, extending the ultrasound time to 50 min resulted in a blue shift, which is attributable to the formation of protein aggregates and inaccessibility of the chromophores (Sun et al. 2020b). In addition, direct ultrasound treatment exerted similar effects on the tertiary structure of protein isolates, such as rapeseed napin (Pan et al. 2020) and buckwheat protein (Jin et al. 2021). For the latter, ultrasonication resulted in a decrease in disulfide bonds, suggesting hydrolytic effects of the process.

11.7.4 Microstructure/Nanostructure

Short-term ultrasound treatment (10 min) during UAE resulted in the formation of arachin (128.33 nm) and conarachin (108.72 nm) particles smaller in diameter than control arachin (190.43 nm) and conarachin (131.46 nm) of peanut protein isolate (Sun et al. 2020b). However, extended exposure to 50 min increased the protein particle diameters of arachin and conarachin to 174.95 nm and 125.31 nm, respectively, negatively impacting the techno-functional properties (Sun et al. 2020b). This effect may be related to the β-sheet conformation induced by extended UAE treatment, which enhances protein aggregation. Similar increases in average particle diameter were observed in the nanostructure of peanut proteins subjected to UAE; however, it is also possible that these effects were due to the partial unfolding of the structure of protein molecules, thus decreasing flocculation and the uniformity in

droplet distribution (Sun et al. 2021). The subsequent increase in particle size is likely caused by the cavitation effect of UAE, which leads to the disruption of internal hydrophobic and electrostatic interactions, and hydrogen bonding (Sun et al. 2021). This effect is confirmed by the observation of large perforations on the rigid external surface of sesame bran post-UAE treatment (Görgüç et al. 2019). This suggests that the ultrasonic vibrations can effectively loosen the cellular structure, thus allowing for a greater penetration of enzyme or solvent during extraction (Görgüç et al. 2019; Görgüç et al. 2020). Conversely, the microstructure of peanut flour protein following UAE showed an increase in particle density but similar particle size diameters compared to the control derived from alkaline extraction (Ochoa-Rivas et al. 2017). This study highlighted the additional effects of other processing parameters, such as spray drying conditions, which play an important role in particle size regulation (Ochoa-Rivas et al. 2017). Lastly, the decrease in microstructure particle size observed with UAE may be because of the effects of cavitation and turbulent forces (Ly et al. 2018). Similarly, fragmentation effects were observed in UAE-treated defatted soy flakes where the prevalence of micro-fractures increased with amplifying ultrasound intensity and exposure time (Karki et al. 2010). This suggests an effect of UAE exposure time and intensity on the effect of protein microstructure that may occur.

11.8 Conclusions

As an emerging technique for protein extraction from different plant sources, ultrasound can improve protein extraction yield by disrupting the plant cell microstructure, and modify protein structures resulting in changes in the physicochemical, functional, and nutritional properties of the proteins. Optimization of UAE conditions is needed for plant proteins from different sources. To date, most of the studies were conducted using laboratory-scale ultrasound equipment (batch type), which generates different results compared to the continuous type and pilot-scale ultrasound equipment. Future studies on pilot-scale ultrasound operations are required in order to facilitate the practical application of UAE for plant protein extraction in the food industry.

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Impact of Green Extraction Technologies on Plant Protein Content and Quality

12

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Abstract

Protein is a vital component of a healthy diet and products derived from plant-based protein sources are being produced and consumed at an increasing rate. The presence of anti-nutritional factors, which limit bioavailability of plant protein, and a lack of sufficient essential amino acids to meet human nutritional requirements are concerning attributes for plant-based protein products and their nutritional quality. Of particular interest are the Protein Digestibility Corrected Amino Acid Score (PDCAAS), an indication of protein and amino acid bioavailability, and the Protein Efficiency Ratio (PER), a growth rate measurement based on quantity of protein consumed. Green methods for processing plant material have been garnering interest from product developers as well as consumers for their reduced environmental impact as well as enhancing the quantity and/or quality of the final protein produced. This chapter presents, where available, a discussion on the content and nutritional quality of plant proteins as determined by PER or PDCAAS following extraction by alkaline extraction and isoelectric precipitation, air classification, high-pressure processing, enzyme-assisted extraction, microwave-assisted extraction, reverse micelle protein extraction, and ultrasound-assisted extraction.

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Keywords

Protein quality · PDCAAS · Protein yield · Amino acid composition · PER

12.1 Introduction

For almost a decade, protein has been the prominent attribute of foods that has supported consumer-driven food innovation. Initially, formulation of high protein food, regardless of the source, satisfied protein's value to consumers, such as ability to enhance feelings of satiety for weight management and muscle accretion (International Food Information Council Foundation 2015, 2018, 2021). While these belief systems remain in place, there has a shift in focus toward the use of innovative plant protein isolates and concentrates to expedite food innovation that appeals to a wider range of the population. This stems from multiple sources, including shifts in dietary guidelines that emphasize consumption of plant protein foods for supporting health and wellbeing, but also global discussions, awareness, and perceptions of animal protein as it relates to environmental degradation animal welfare Charlebois et al. 2018. Consumer's increasing desire to shift their dietary patterns to include more plant protein has caused significant transitory period for the global food industry. New technologies continue to be developed at unprecedented speed for supporting a wide range of innovative plant-derived protein foods that appeal to a greater swath of the population.

However, as dietary patterns include more plant protein from manufactured foods, one cannot forget that, biochemically, protein is an aggregate of amino acids, of which there are nine indispensable amino acids (IAA) required to satisfy human's requirements for endogenous protein synthesis, metabolism, and growth and/or maintenance. Thus, while food innovation enhances the use of new technologies to derive functional plant protein ingredients, the protein quality of these ingredients also requires consideration, especially if foods are to successfully achieve protein nutrient content claims in Canada (Government of Canada 2020) and the USA (Government of the United States of America 2018).

The concept of protein quality is not new. For example, Osborne et al. were the first to discuss the protein efficiency ratio method in 1919 Osborne et al. 1919, as a method to "demonstrate the maximum power of any protein to promote growth." Since then, more accurate assessments of protein quality have been developed for human foods that can be aligned with the indispensable amino acid requirements of humans across the life stage. These methods include the Protein Efficiency Ratio (PER) (HealthCanada 1981), protein digestibility corrected amino acid score (PDCAAS) and Digestible Indispensable Amino Acid Score (DIAAS) (FAO 2013; FAO and WHO 1991). Rather than a direct analysis of amino acid composition, the PER is a measurement of growth per unit protein consumed as determined in a rodent model (HealthCanada 1981). Detailed information on deriving PDCAAS and DIAAS from foods are described elsewhere (Marinangeli and House 2017). Briefly, both methods rely on the indispensable amino acid composition of a food,

and indispensable amino acid requirements (mg IAA/g protein) of a reference population. For regulatory frameworks, the reference pattern for children is typically used because IAA requirements are higher. The underlying difference between PDCAAS and DIAAS is the use of true N digestibility and ileal IAA digestibility values, respectively. While DIAAS is considered a more accurate representation of protein quality, and was developed to address many of the shortfalls of PDCAAS (Marinangeli and House 2017), PDCAAS remains in widespread use and is the framework for protein nutrient content claims in the USA (Government of the United States of America 2018, 2020) and an option in Canada (Government of Canada 2020). Generally, protein's derived from plants have a lower protein quality compared to animal-derived proteins. This is typically due to lower levels of one or more IAA relative to requirements in the reference pattern, and, in some cases lower digestibility values (Tome 2013).

One of the challenges facing widespread utilization of plant-based protein is extracting the protein from the plant matrix itself. This task has encompassed a wide variety of techniques ranging from extraction based on physical density, air classification, to more modern extraction protocols using reverse micelle or ultrasound-assisted extraction of the plant protein fraction. Each of these methods presents unique challenges and opportunities for generation of plant-based protein for use in novel product development. This chapter will provide an overview of certain extraction technologies, their impact on protein content, amino acid composition, and protein quality itself where those data are available. Specifically, alkaline extraction coupled to isoelectric extraction, air classification, high-pressure processing, enzyme-assisted extraction, microwave-assisted extraction, reverse micelle protein extraction, and ultrasound-assisted extraction will be discussed.

12.2 Protein Extraction Methodologies

12.2.1 Alkaline Extraction and Isoelectric Extraction

Alkaline extraction with isoelectric precipitation (AEIP) is the most common method applied to produce plant protein isolates of 80–≥90% protein. It is a wet extraction technique, whereby milled raw materials are suspended in water. Depending on material and solubility of proteins, the pH of the solution is titrated to 8–11 using a base, such as NaOH, to solubilize albumin and globulin fractions Barac et al. 2015. After centrifugation, the protein-rich supernatant is collected and the pH of the solution is lowered to the isoelectric point of proteins, which causes their subsequent precipitation. Centrifugation is used to isolate and collect the protein-rich pellet Barac et al. 2015. The isoelectric point can differ between the types of protein, which can affect the milieu of proteins collected. In addition, varying the conditions during the extraction process can cause proteins to complex with other proteins or compounds. Isoelectric precipitation is most effective at precipitating globulin protein fractions, with most albumin protein remaining in solution (Barac et al. 2015; Emkani et al. 2021).

As a common method for producing isolated protein fractions, most studies on AEIP have focused on deriving protein fractions with optimal functional and/or hedonic properties for food innovation. Few studies have investigated the AEIP conditions on parameters related to protein quality of protein ingredients, such as digestibility, and indispensable amino acid concentrations. Studies have aimed to use adjunctive technologies to enhance extraction efficiencies and protein yields of AEIP, which could affect protein quality by increasing total protein and/or composition of proteins in the final product. This could affect protein quality by modulating indispensable amino acid profiles and digestibility coefficients of the final product. For example, Emakani et al. Emkani et al. 2021 compared the use of HCl or lactic acid with fermentation by either *Streptococcus thermophilus, Lactobacillus acidophilus, or Bifidobacterium lactis* to acidify and precipitate alkali extracts of yellow pea flour. Results demonstrated that nitrogen from protein was higher in globulins protein precipitated by HCl and lactic acid at 16% protein N compared to 9.5–14.5% protein nitrogen from fermented samples. Conversely, albumin levels that remained in solution did not differ in N protein levels across acidification protocols. It was hypothesized that proteolytic enzymes during fermentation, combined with acidification, could have caused a decrease in protein from the globulin fractions Emkani et al. 2021. While the peptide profile did not differ across methods of isoelectric precipitation, it is reasonable to infer that the loss of protein following lactic acid fermentation could affect protein quality if there are substantial losses of indispensable amino acids.

Given that protein components can vary in structure, optimization could yield enhancements or reductions in protein quality of an extracted ingredient. The various globulins of pea protein, for example, differ in molecular structure, with legumin recognized as having higher levels of sulphur amino acids compared to vicilin and convicilin. The provision of sulphur amino acids is a recognized target for protein complementarity when combining plant protein ingredients to increase the protein quality of manufactured plant protein foods (Nosworthy and House 2017). Gao et al. Gao et al. 2020 demonstrated that increasing pH 8.5–9.0 and 9.5 enhanced the protein extraction yield and protein recovery from yellow peas from 12.93% and 49.20% to 14.00% and 52.43%, and 15.36% and 57.56%, respectively. Although the profile proportions of convicilin, vicilin, and legumin did not differ across pH values, higher levels of protein aggregation were demonstrated when proteins were extracted at pH 9.0 and 9.5 compared to pH 8.5 due to increasing free sulfhydryl and disulfide bonds, which decreased solubility (Gao et al. 2020). Solubility has been shown to decrease protein digestibility, which inversely affects protein quality Carbonaro et al. 1997. Similar results from Poten et al. Potin et al. 2019 found that extraction yield of hemp protein significantly increased from ~6% at pH values of 2–7 to 67% at pH 12, with a concurrent shift toward extraction of globulin proteins. Alongside higher extraction yields, subjecting protein to extreme alkali conditions caused protein aggregation, including complexes with concurrently extracted phenols Potin et al. 2019, which could affect protein digestibility and subsequent amino acid bioavailability Sęczyk et al. 2019.

AEIP is the most common method applied to create highly concentrated protein extracts with the flexibility to optimize the extraction parameters for the isolation of proteins that meet the functional needs within food products. However, adjustments to pH and use of methodological adjuncts may affect protein content, structure, and composition that could affect protein quality.

12.2.2 Air Classification

Air classification uses cyclonic air flow and centrifugal and centripetal forces to separate and concentrate starch, fibre, and protein components of milled commodities. During air classification, protein fractions concentrate in fine and ultra-fine particles Rempel et al. 2020; Tyler et al. 1981). Generally, air classification is less effective than wet protein extraction methods, such as AEIP, and produces protein concentrates ranging from 50 to <80% depending on the ingredient and method optimization. For example, air classification has been shown to produce protein concentrates of 38–68% protein in lentils Khazaei et al. 2019, 50–70% protein in faba beans (Gunawardena et al. 2010; Saldanha do Carmo et al. 2020; Schutyser et al. 2015; Tyler et al. 1981), and 45–60% protein in peas (Gunawardena et al. 2010; Nosworthy et al. 2017; Pelgrom et al. 2013; Tyler et al. 1981). Methodological optimization for air-classified canola meal was able to increase the concentration of protein in the fine fraction 10.7%, an increase of 39.2 g (365 g protein/kg to 404.2 g/kg) (Rempel et al. 2020).

Few studies have examined the protein quality of air-classified protein ingredients. Given that protein concentrates from air classification have lower levels protein compared to wet extraction methods, the presence of anti-nutritional factors, or residual levels of other constituents, such as fiber, could decrease the digestibility of fractions. Conversely, dry classification of protein does not discriminate against various protein fractions in the same way as wet methods, such as AEIP, resulting in lower losses of IAA. This was demonstrated by Vogelsang-O'Dwyer et al. (Vogelsang-O'Dwyer et al. 2020) and Nosworthy and House (Nosworthy and House 2017), where air-classified faba bean and pea protein concentrate produced higher amino acid scores (faba bean concentrate: 0.62; pea concentrate: 0.58) compared to wet extracted protein isolates (Faba bean isolate: 0.53; pea isolate: 0.54). For pea, this translated into a PDCAAS that was 2.5% higher for pea concentrate compared to pea isolate (M. G. Nosworthy and House 2017). Disparities in amino acid scores was hypothesized to be lower levels of sulphur amino acids (methionine + cysteine) in the protein isolate from loss of specific sulphur-rich proteins during the extraction process (Vogelsang-O'Dwyer et al. 2020). However, in the same study by Vogelsang-O'Dwyer et al., isolated faba bean protein demonstrated 91% lower trypsin inhibitor per mg protein and a 3–5% increase in in vitro digestibility compared to a faba bean protein-rich flour as prepared by air classification (Vogelsang-O'Dwyer et al. 2020).

While air-classified ingredients typically have lower levels of protein compared to isolates, methods can be modified to enhance protein quality. Hydrolysis of

air-classified pea protein (49% protein) with trypsin, savinase, or papain decreased levels of phenolics (13–37%) and tannins (100%), and the activities of trypsin (55–74%) and chymotrypsin (89–94%), all of which can affect the digestibility of proteins (Konieczny et al. 2020). Although enzyme type and degree of hydrolysis had differential effects on each outcome, in vitro digestibility of treated pea protein concentrate increased 2–7%. However, 2–4% hydrolysis for all enzymes decreased the amino score from 0.79 to 0.69–0.75, from losses of cysteine during the hydrolysis process. On the other hand, 10–12% hydrolysis only modestly decreased cysteine levels and increased levels of methionine 25–36%, which left the amino score unchanged or increased from 0.79 to up to 0.84 (Konieczny et al. 2020). Altogether, results of this study on physiochemical characteristics of pea protein concentrate had differential effects on the in vitro PDCAAS, which decreased or increased from 66.68 in untreated pea protein concentrate from 59.17 to 72.89, respectively (Konieczny et al. 2020). Similar results were demonstrated when pea protein concentrate was fermented with *Aspergillus oryzae* and *Aspergillus niger* for 2, 4, and 6 h, where, despite reductions in trypsin and chymotrypsin activities by up to 30.4% and 21.8%, respectively, the amino acid score was decreased 4–14% and the in vitro digestibility 5–15% (Kumitch et al. 2020). These latter effects reduced the PDCAAS of pea concentrate by 5–15% across fungi and all fermentation periods (Kumitch et al. 2020). Similar to other processes, air classification can be used to generate protein ingredients with modest levels of protein. The quality of protein produced by air classification can be optimized through the manipulation of equipment parameters or exposing ingredients to various treatments post-extraction.

12.2.3 High-Pressure Processing

Once considered unique, high-pressure processing has become more ubiquitous method for improving the functionality of ingredients within food platforms. In fact, in 2016, Health Canada removed high-pressure processing as a novel process, given its use in commercial settings and little-to-no evidence of negative effects on foods safety (Health Canada 2016). In plant-derived protein foods, high pressure is often used to control enzymatic degradation of food as well as destroy anti-nutritional factors (Gharibzahedi and Smith 2021). Furthermore, as a non-thermal process with a high degree of customization, it can be applied to foods with controllable effects on functionality and hedonic properties. Effectiveness of high pressure to modulate the properties of protein in food is a function of type of protein, pH, ionic strength of proteins, and the intensity of pressure applied to the food system (Gharibzahedi and Smith 2021).

Given the pressure can affect the structure of proteins, it is reasonable that protein quality could be increased by way of enhancing levels of free amino acids and/or affecting protein digestibility. However, conditions can produce mixed effects on amino acid and total protein levels. When brussel sprout seedlings were exposed to 200–800 kPA pressure for 3 min at 5 °C, there was small increase noted in levels of free glutamine and aspartate, alongside minor decreases in alanine, glycine, leucine,

phenylalanine, proline, serine, tryptophan, and tyrosine decreased Barba et al. 2017. While it was speculated that exposure to pressure was insufficient to cause major changes in amino acid levels, enzymatic activation was hypothesized to facilitate conversion of glutamine and asparagine to glutamate and aspartate, respectively Su-Yeon et al. 2014. In a similar study, root vegetables, leaf vegetables, and pulses were subjected to 20 MPa for 20 min at 125 °C in an autoclave. High pressure, high-temperature processing decreased levels of lysine and arginine of vegetables and decreased lysine, arginine, and cysteine of pulses (Su-Yeon et al. 2014). However, when soy slurry was subjected to high-pressure homogenization at 100 MPa, the protein extraction yield increased from 65% to 82% Preece et al. 2017. However, multiple exposures to pressure treatment caused a stepwise decrease in protein extraction yield to baseline levels after 5 passes, due to a reduction in protein separation efficiency from the swelling of cell walls Preece et al. 2017. As with other methods, these results highlight the importance of method optimization when using high pressure to enhance factors that affect protein quality.

Enzymatic hydrolysis of proteins is a well-known process to increase the functional properties of proteins. Enzymatic hydrolysis can also increase the protein quality of food proteins by generating smaller peptides that are more digestible by digestive enzymes. High-pressure processing has been shown to enhance enzymatic hydrolysis of food proteins. For example, using high-pressure homogenization under 0.1, 40, and 80 MPa caused a stepwise increase in peanut protein extraction yield of 16.84%, 30.65%, and 39.86%, respectively Dong et al. 2011. In addition, when used in combination with the proteolytic enzyme Alcalase, high-pressure homogenization at 40 and 80 MPa increased degree of hydrolysis (Dong et al. 2011). Similar results were demonstrated in defatted rice bran where, amylase, in combination with the application of 500 and 800 MPa (5 min at 25 °C) increased percent extraction yield to 35.8 and 37%, respectively, compared to 33% when 0, 200, and 500 MPa of pressure was applied (Dong et al. 2011). However, there was no enhancement in protein extraction when rice bran was treated with protease Tang et al. 2002. It is hypothesized that treatment of proteins with pressure helps to unfold the proteins to expose sites for enzymatic hydrolysis (Gharibzahedi and Smith 2021). In the same regard, amylase from the Tang et al. Tang et al. 2002 may have helped release proteins bound to starch, and, combined with pressure, improved protein extractability. Although these studies suggest that high-pressure processing could enhance protein digestibility in relation to protein quality, the effects remain to be elucidated in a biological system.

12.2.4 Enzyme-Assisted Extraction

Enzyme-assisted extraction relies on the specificity of enzymes to disrupt cell walls via hydrolysis Nadar et al. 2018. In brief, the binding of the enzyme to the cell wall induces a conformational change in the enzyme resulting in the breaking of molecular bonds in the cell wall leading to the release of cellular material, including the protein fraction. As temperature and pH are essential variables for the optimization

of enzymatic activity, there are a wide range of enzymes used in the extraction of plant proteins. Among others, these include cellulase, glucoamylase, papain, pectinase, and xylanase (Nadar et al. 2018; Puri et al. 2012). When compared to other traditional extraction methods, enzyme-assisted extraction has demonstrated lower requirements for energy inputs and solvent usage, while maintaining overall protein yields Puri et al. 2012.

Considering oilseeds, such as canola, there is a growing interest in identifying novel protein extraction techniques that do not rely on the use of solvents due to health and environmental concerns. When comparing traditional solvent extraction to aqueous enzyme-assisted extraction the amount of extracted protein was significantly higher when solvents were used—37.2% compared to 12.4–15.1% Latif et al. 2008. A comparison between four different enzymes, Protex 7 L, Multifect Pectinase FE, Multifect CX 13 L, and Natuzyme illustrated the importance of enzyme selection as the Protex 7 L generated 12.4% protein, significantly lower than the other enzymes investigated (Multifect Pectinase FE 14.5%; Multifect CK 13 L 15.1%; Natuzyme 14.8%). Similar results were obtained when comparing different enzymes in the extraction of protein from soy grit, coarsely ground soy flour Perović et al. 2020. Pectinase and cellulase increased protein yield by ~10–12% to 25% and 27%, respectively, while a combination of 0.7 U pectinase, 4 U xylanase, and 20 FPU cellulase increased yields by ~20% to 35%. Interestingly, when the amount of enzymes in the mix was altered, 0.4 U pectinase, 33 U xylanase, and 20 FPU cellulase, the extraction yield was reduced to ~17%. This highlights the fact that while enzymes can have a synergistic effect related to protein extraction, optimization of the mixture can have dramatic effects on overall yield.

In extracting protein from sugar beet leaves, the use of Pectinex Ultra SP-L enzyme was able to increase the protein yield from 34.55% to 79.01% through the breakdown of the glycolytic bonds in the cell wall (Akyüz and Ersus 2021). *Moringa oleifera* leaves contain 22.2–33.4% protein, however, extraction through traditional means can be disruptive to the nutritional quality and digestibility of the protein Benhammouche et al. 2021. Protein extraction from defatted *M. oleifera* leaves by an enzyme cocktail, Viscozyme L, increased protein yield by >10% over alkaline extraction, with the protein digestibility being 64.7%. Interestingly, the PDCAAS of the protein extract was approximately 64.7, as the amino acid score was determined to be >1. A protein concentrate produced from these leaves had a PDCAAS >90 as the digestibility of the protein concentrate was almost 100%.

Enzyme-assisted extraction can also have direct influence on amino acid composition, and subsequent taste experiences, such as umami. A study conducted on different mushroom species determined that a combination of β-glucanase and Flavourzyme significantly increased the release of MSG-like amino acids compared to standard HCl extraction Poojary et al. 2017. This study also did extensive comparisons between pH (4–7), temperature (30–60 °C), and enzyme concentration(0.1–5% w/v β-glucanase, Flaourzyme, or combined), resulting in the optimal conditions of %5 w/v of the combined enzymes, pH of 7 at 50 °C for 1 h.

These examples of enzyme-assisted extraction serve to demonstrate the applicability of this technology, as well as its potential. By altering time, temperature, pH,

and enzymes selection, protein can be extracted from difficult substrates such as leaves and oilseeds, while still maintaining yield and characteristics valued by consumers and product developers.

12.2.5 Microwave-Assisted Extraction

Microwaves have been a staple in many kitchens for rapid food preparation for decades, however their use in protein extraction from plant matrices is relatively recent. The penetrative ability of microwaves, due to their electromagnetic wave frequencies between 300 MHz and 300 GHz depending on application, increases their efficiency both in food preparation and protein extraction (J. Tang 2015). Microwave-assisted extraction (MAE) of proteins, or other biological compounds, from plant matter relies on the destruction of cell walls subsequent to the rapid expansion of water present in the cells induced by molecular frictions caused via dipole polarization Flórez et al. 2015. This disruption then releases the compounds of interest into an appropriate solvent, frequently water, from which the desired chemicals can be reclaimed for further purification or processing. Due to its lower energy requirement and potential for green solvent use, this processing method has been applied to protein extraction from a wide variety of plant sources including rice bran, peanut flour, sesame bran, and sunflower cake (Görgüç et al. 2020; Náthia-Neves and Alonso 2021; Ochoa-Rivas et al. 2017; Phongthai et al. 2016). While similar in some ways, the specifics of the MAE process do vary depending on the starting material.

Sunflower is one of the primary oil crops in the world, with the majority of sunflower cake produced after oil extraction being discarded or used for livestock feed. This cake is protein rich, 27–63%, however the high quantity of phenolic compounds has prevented widespread use in novel foods. Use of MAE for extraction of phenolics from sunflower cake did not see any change in protein content post-processing (27% vs 26%) (Náthia-Neves and Alonso 2021). Conversely, MAE did increase protein yield extracted from sesame bran (62.3%) compared to that extracted by standard alkaline extraction (24.5%) Görgüç et al. 2020. The purity of protein extracted by MAE is also high, as demonstrated using peanut protein where a purity >90% was achieved using MAE Ochoa-Rivas et al. 2017.

While protein content is an important factor when considering extraction methodology, amino acid composition must also be considered. In sunflower cake, the fraction of most amino acids present per gram of raw material was increased following MAE, ranging from an increase of 3.7% for histidine to 66% for proline (Náthia-Neves and Alonso 2021). A similar result was determined in broccoli, where amino acid extraction was compared between a shaking method and MAE Kovács et al. 1998. While total protein was not assessed in that study, all amino acids extracted except phenylalanine, tyrosine, and arginine were in higher concentration following MAE. As essential amino acid content is a primary factor in protein quality assessment, these results indicate that use of MAE could result in a protein fraction of greater nutritional value.

12.2.6 Reverse Micelle Protein Extraction

The reverse micelle has been used in multiple applications including encapsulation of water soluble antioxidants, oil extraction, purification of enzymes, and general extraction of proteins from plant-based sources (Xiaohong Sun and Bandara 2019). The compounds extracted are highly dependant on the surfactant being used in the process, although sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and cetyltrimethylammonium bromide (CTAB) are commonly used for protein extraction. The process by which this method extracts plant proteins is a two-step process, a forward extraction followed by a reverse extraction Leser et al. 1993. In the forward extraction the proteins are solubilized into the reverse micelle, while the subsequent reverse extraction recovers the solubilized protein. This method has been used for extraction of a diverse set of plant proteins including wheat germ (Sun et al. 2008; Zhu et al. 2010), soybean (Zhao et al. 2018), and walnuts (Wang et al. 2021), as well as for amino acid extraction (Bayraktar et al. 2008), however much of the work has been done on optimizing either the forward or reverse extraction rather than total protein yield.

The protein content of defatted wheat germ (DWG) is over 30% making it a good target for protein extraction, while its amino acid composition highlights its potential as a high-quality protein source (Ge et al. 2000). One investigation of protein extraction from DWG determined a forward extraction efficiency of 37% under optimal conditions and a backward extraction efficiency of 80% for a final extraction efficiency of 30% (Sun et al. 2008). Further work by the same group found the same extraction efficiency (30%), with the protein content of the extracted sample increasing to 81.63% from an initial protein content of roughly 30% Zhu et al. 2010. In soybean, protein yields have reached 72.4% with final protein contents of 82.5–83.6% (Zhao et al. 2018; Zhao et al. 2015). For extraction of walnut proteins, the backwards extraction efficacy is frequently lower (60%) compared to other plant sources such as soy (90%) and wheat germ (80%) Wang et al. 2021. This necessitates the use of multiple techniques such as microwave-assisted backwards extraction, which increased the extraction yield from 57% to 95%.

While less understood than extraction efficiency, a few studies have been conducted investigating the amino acid profile of plant proteins post-extraction. Total soybean amino acids extracted by the reverse micelle method were greater than isoelectric precipitation (82.5% vs. 79.3%) (Zhao et al. 2018). This includes significantly greater content of multiple amino acids such as lysine, methionine, tyrosine, and phenylalanine. Similar results were found with DWG where the relative amino acid profile following reverse micelle extraction was typically higher than that of isoelectric precipitation, including lysine, total sulfur amino acids (cysteine, methionine), threonine, and histidine (Zhu et al. 2010). Similar to previously discussed extraction methods, there is a lack of information directly related to protein quality assessment. However, one study did determine the amino acid score of reverse micelle extracted protein to have an amino acid score of 115, and a PER of 2.4 via mathematical modeling (Zhao et al. 2018).

12.2.7 Ultrasound-Assisted Extraction

The term ultrasound refers to sound waves above the range of human hearing, with a frequency between 20 kHz to 1 GHz (Musielak et al. 2016). Within this range, frequencies of 20–100 kHz are those commonly used for food processing (Gençdağ et al. 2021). At these frequencies, the alternation of compression and rarefaction that occur as the wave propagates through a liquid medium results in the generation of bubbles and their subsequent collapse—an event known as cavitation. This cavitation can generate temperatures up to 5000 K and pressures upwards of 1200 bar (Kentish and Feng 2014).

This generation of localized changes in the temperature and pressure results in generation of shear forces via macroturbulence, leading to the subsequent disruption of the cell wall and release of cellular components such as protein. This process has been used either alone, or in conjunction with other extraction methods such as alkaline extraction, to extract protein from multiple plant sources. This includes, among others, soybean sprouts (Yang et al. 2015), sesame cake (Yang et al. 2021), *Moringa oleifera* leaves (Cheng et al. 2021), brewers spent grain (Tang et al. 2010), peanut protein isolate (Sun et al. 2021), as well as pulse and soy flours (Byanju et al. 2020).

Unsurprisingly, the optimization of protein extraction via ultrasound results in differences of power levels, timing of ultrasound exposure, and the utilization of combinations of extraction techniques. In the case of soybeans, exposure to ultrasound at 200 W or 300 W prior to sprouting significantly increased the protein content compared to no ultrasound exposure (47.6%, 47.7%, and 45.7%, respectively) (Yang et al. 2015). Disruption of lignans in sesame cake using an ultrasound pre-treatment also resulted in increased protein extraction (K. Yang et al. 2021). In this study, the final protein content extracted from cold-pressed sesame cake using isoelectric precipitation was significantly greater following exposure to ultrasound frequencies (22.2% vs. 26.0%). The leaves of *Moringa oleifera*, consumed in many tropical and subtropical regions, have a protein content ranging from 6.7–29.4% depending on whether they are fresh or dried (Dhakad et al. 2019). The protein content extracted from *M. oleifera* leaves using different extraction parameters ranged from 63.8 mg/g to 82.4 mg/g, greater than that of traditional solvent extraction (Cheng et al. 2021). Ultrasound protein extraction from brewers spent grain, a by-product of the brewing process with protein content of approximately 20%, extracted 96.4 mg/g of protein similar to the maximum predicted by mathematical modeling Tang et al. 2010. For peanut protein isolate, the maximum ultrasound protein extraction efficiency was 91.4%, compared to an efficiency of 77.6% for alkali extraction (Sun et al. 2021). In the case of kidney bean and soy flake flours, power density of 2.5 or 4.5 W/cm^3 of the ultrasound extraction altered the extraction yield Byanju et al. 2020. A significant increase in protein yield for ultrasound extraction of soy flake was determined at 2.5 W/cm^3 (68.5% increase) and 4.5 W/cm^3 (90% increase). A 16.4% increase in extraction yield was noted for kidney bean flours at 4.5 W/cm^3, however, no significant difference was found at either power density for soy flours, and overall protein extraction yield decreased with increasing

power density for chickpea flour, albeit insignificantly. These studies demonstrate the potential for ultrasound extraction of plant proteins either directly, through a pre-processing step, or in conjunction with other techniques however the alteration of final amino acid composition must be considered as well.

In general, the use of ultrasound in protein extraction results in an increase in amino acid quantity (Trakselyte-Rupsiene et al. 2021; Wang et al. 2020), most likely due to the increase in protein yield described above. Soybean sprouts were found to have an increased amino acid content post-exposure to ultrasound (Yang et al. 2015). Specifically, at 100 or 200 W, ultrasound treatment increased the content of all essential amino acids, as well as Gly and Ala, in soybean sprouts. Similar results were found in peanut protein isolate, where the essential amino acid content was higher following ultrasound extraction (Sun et al. 2021). Interestingly, while there was no difference in total amino acid content after 10 or 50 min of ultrasound treatment compared to control (94.8%, 92.8%, and 93.2%, respectively), exposure for 30 min resulted in significantly lower total amino acid content (89.9%). Ultrasound treatment has also increased the total amino acid content extracted from walnut protein isolates Golly et al. 2020. This increase in amino acid content may be matrix dependent or reliant on optimization however, as no difference in amino acid composition was noted when sesame press cake was exposed to ultrasound (K. Yang et al. 2021). Unfortunately, there is limited information available on the digestibility of proteins produced via ultrasound-assisted extraction, however the increased protein content, and higher content of essential amino acids, in extracted samples suggest that this method would be useful in the generation of high-quality protein products.

12.3 Conclusions and Future Directions

The increasing demand for alternative protein sources, such as those derived from plants, is driven by consumers desire to have dietary options that are healthier for them as well as the environment. While some methods such as alkaline/isoelectric precipitation and air classification have been well established and their ability to purify plant proteins characterized, in order to satisfy this demand for quality protein while maintaining environmental sustainability, development, and optimization of greener methods for protein extraction is being conducted. These more recent innovations include high-pressure processing, enzyme-assisted extraction, microwave-assisted extraction, reverse micelle protein extraction, and ultrasound-assisted extraction. As described in this chapter, these methods are capable of extracting protein of high purity and significant yields depending on the starting material, whether it be ground flour or purified protein concentrates/isolates. There are, however, concerns regarding these processing methods that require further investigation.

In particular there is a dearth of data related to the amino acid composition and in vivo, or in vitro, digestibility of the proteins produced by these methods. Total protein yield is a valuable detail but without accurate determination of the protein

quality, as identified by PER/PDCAAS/DIAAS, placing these extracted proteins in a nutritional and regulatory context becomes impossible. Furthermore, most of these methods are also performed at a lab-scale rather than at the high throughput that is required by industry is also worth noting, as optimization on a small scale does not always translate to larger scale extraction protocols. Overall, however, the presented data indicates that these methods are valuable additions to the plant protein extraction space and with further development their widespread use will be of great benefit to novel product development in an environmentally friendly fashion.

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Effects of Extraction Technologies on the Functionalities and Applications of Plant Proteins

13

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Abstract

The need to provide proteins to a rapidly growing world population calls for a sustainable plant-based protein supply. There has been a growing demand to concentrate and isolate proteins from plant resources as alternatives to meat and milk, or traditional animal protein ingredients to provide structure and texture of a wide range of food products such as baked goods, beverages, and snacks. In response to this demand, novel technologies are required that can more efficiently extract proteins from plant sources while maintaining or even improving their sensory quality, functionality, and nutritive value. This chapter has reviewed both conventional and novel technologies that have been applied or under exploration for plant protein extraction and isolation from pulse and cereal crops. The innovative protein isolation methods are highlighted such as emerging solvent, energy-assisted, and enzyme-assisted extraction methods. The effects of the extraction technologies on the plant protein molecular structures and functional properties are emphasized. The food applications of the plant protein ingredients are illustrated.

Keywords

Extraction technologies · Plant proteins · Structure · Functionalities · Food applications

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13.1 Introduction

The consciousness of climate change, ever-growing world population, and food security challenges has encouraged the search for sustainable protein ingredients to obtain affordable and nutritive food products. This trend has inspired academic and industrial communities to explore toward new and diversified sources to generate protein ingredients as alternatives to those from traditional animal-based sources such as dairy and meat products. In recent years, plant protein-based ingredients and food products have taken the center of the stage in product innovations in food industry. Cereals (wheat, rice, corn, barley, and oat) and legumes (peas, beans, lentils, soybean, lentils) are important parts of diets and contribute substantially to the nutrient intake of human beings. Cereals and legumes have attracted significant interest in protein ingredients manufacturing because of their large production, wide accessibility, health-promoting benefits, and sustainability as compared to animal sources.

Currently, wet extraction methods are applied to obtain plant protein concentrates or isolates. Among them, alkaline extraction followed by isoelectric precipitation is widely used (Karaca et al. 2011). Owing to their high purity, protein isolates by wet methods generally have good functional properties such as foaming, emulsifying and gelling properties, thus are widely used in food formulars as functional ingredients to provide food structure and texture. On the other hand, dry methods such as air-classification are used to separate protein particles from starch and fibers to obtain protein-enriched flours. Advantages, including the low consumption of water, no requirement of stabilization process after separation and readily scalable for potential applications, allow air classification to be one of the most popular techniques to obtain plant proteins from pulses and cereals. The protein content can be increased to about 50–60% for pulses such as pea, mung beans, faba bean, and lupine bean, and about 25% for cereals such as wheat, barley, and rich bran (Zhu et al. 2021).

Though conventional protein extraction methods have been commercially applied to obtain plant protein products, such as soy protein and pea protein, these methods have limitations that hinder the development and growth of plant protein industry. For example, air classification has low efficiency when protein and other components have similar particle size and/or density. Wet extraction techniques generate a large amount of water waste with alkali and salts. The commonly used alkali extraction method followed by isoelectric precipitation is effective to extract proteins from soy meals under mild alkaline pH. For many other plant sources, alkaline extraction is not as efficient, as a result, higher pH (~10–11) and/or heating is used, but this causes protein denaturation and possibly reduces functionality, sensory quality, and nutritive value (Momen et al. 2021). This is because proteins are confined within the plant matrix, not only the cell wall but also the interaction between protein and other components such as polysaccharides and polyphenols may hinder protein extraction, leading to a relatively low extraction yield. Thus, the current extraction technology is not sufficient to dissociate those interactions and hardly increase protein recovery and purity. Therefore, new techniques are waiting

to be developed for improved protein extraction from pulse, cereal, oilseed and pseudocereal crop.

To address the challenges mentioned above, innovative technologies have been emerging for protein processing from cereals and legumes, such as energy-assisted techniques (high pressure, microwave, pulsed-electrical field, ultrasound) and enzyme-assisted techniques. Based on the processing conditions applied during extraction, such as solvent, energy input, and enzyme hydrolysis, these new techniques profoundly impact protein functionality by modifying their primary structure, conformation, and aggregation. The current challenge is to understand how the changes in protein functionality correlate with structural changes enabled by the novel techniques. Figure 13.1 illustrates the various extraction technologies and applications discussed in this chapter. In recent years, an increasing number of studies have shed interesting light on the protein molecular structure modifications enabled by both traditional and novel protein extraction and other processing technologies. These have allowed us to have a more-in-depth discussion of how the protein extraction technologies impact the molecular structures, functional properties, and sensory quality of plant protein ingredients from cereal and legumes. In addition, the academic and industry communities have assessed and expanded the applications of plant proteins in food and non-food products as new functional ingredients, which are also illustrated in this chapter.

13.2 Dry Processes

The plant protein extraction methods could be classified into dry and wet processes. The dry processes are believed to be more environmental-friendly and sustainable due to its high energy efficiency and low water consumption (Schutyser and van der Goot 2011). Moreover, the impacts of dry processes on the protein structure are milder and gentler, allowing plant protein to maintain its native structures. The dry process techniques include milling, air classification, and electrostatic separation. Despite these advantages, there are still some challenges that restrain the potential of dry processes, such as relatively lower protein purity and controlling off-flavors. The advantages, limitations, and future research perspectives of dry processes are discussed in this session.

13.2.1 Air Classification

Milling and air classification is the most common dry process technique to produce protein and starch-rich flours from high-starch cereal or legumes. Flour is firstly finely milled and then air-classified. Milling of the flour makes the large starch granules (2–40 μm) detached from the smaller protein-rich particles (1–3 μm), which allows further separation through air classifying. Then the finely milled flour is suspended in air stream and fed into the air classifier. The air flow is generated by vacuum or centrifugal motion with the assistance of a vacuum. The induced

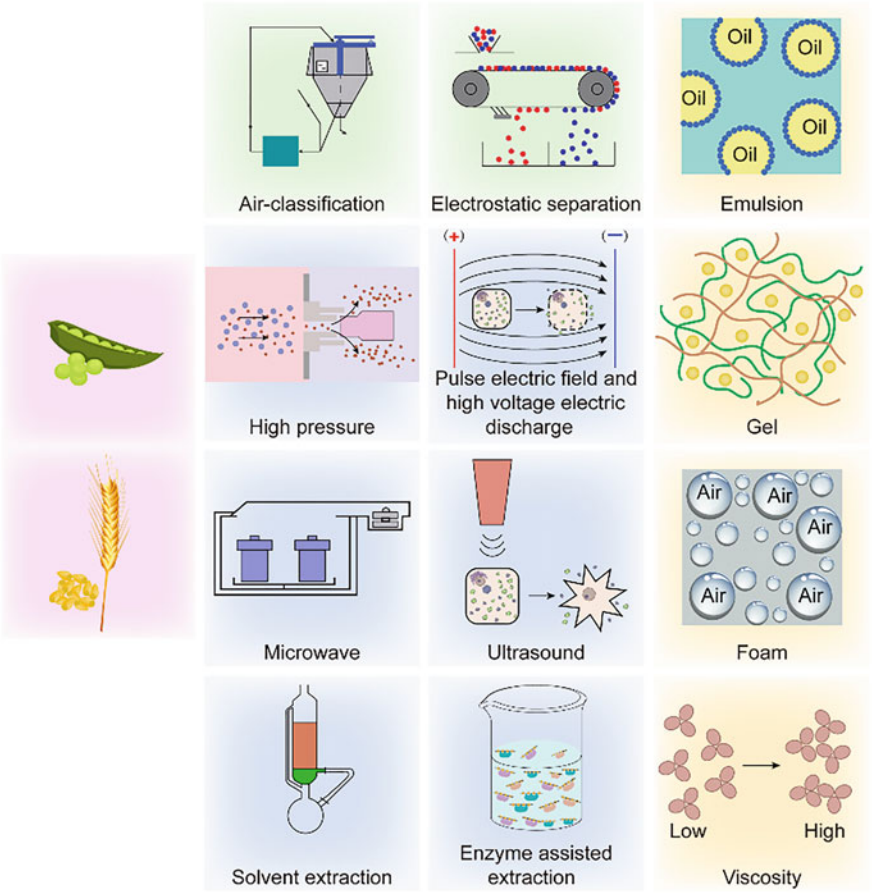

Fig. 13.1 Extraction methods of plant proteins and their functionalities. The extraction methods include dry and wet processes, depicted with green and blue background, respectively. The dry processes include air classification and electrostatic separation technologies. The wet processes include conventional and novel solvent extraction methods, energy-assisted extraction technologies (high pressure, pulse electric field and high voltage electric discharge, microwave, ultrasound) and enzyme-assisted extraction method. Various protein functionalities (depicted in yellow background) were discussed in this chapter, such as emulsification, gelling, foaming, and protein solution viscosity

centrifugal and gravitational forces inside the air-classifier chamber separate the flour based on their density and particle size into two fractions. The fine fraction has a particle size primarily below a targeted particle size and the coarse fraction has larger than this size. The targeted particle size is the optimum setpoint, depending on the size of most starch granules and for the best outcome of protein and starch separation. For example, the optimum setpoint for most of the pulse protein-starch separation is around 20 μm (Fernando 2021). In general, the protein particles have

smaller size and are enriched in the fine fraction while the dietary fiber and larger starch granules segregate into the coarse fraction (Fernando 2021). Air-classification has been applied on pulses, such as pea, mung bean, faba bean, and cereals such as oats and oilseed meals (Zhu et al. 2021). The fine fraction has protein content ranging from 25 to 75%, depending on the seed, environmental factors, and processing conditions (Zhu et al. 2021). For example, it has been reported that by using industrial-scale air classification, 85–87% of protein from the parent pea flour could be concentrated in the pea fine fractions, leading to a pea protein flour with significantly increased protein content (about 50%) (Rempel et al. 2019). Sibakov reported that an oat protein concentrate with a protein content of 73% could be obtained by using air-classification three times to separate protein from starch and dietary fibers. There was 17% of starch in the protein-enriched fraction, but neither β-glucan nor arabinoxylan was detected (Sibakov et al. 2011). This work also pointed out that lipid removal by supercritical carbon dioxide (SC-CO_2) technique was important for enhanced separation of oat grain cell wall material from starch and protein. This is because the milling efficiency increased after lipid removal. More starchy materials were released from cellular structure and into smaller particles, which can be better separated from the cell wall materials, mainly β-glucan. However, since the air classification mechanism largely depends on particle size and density, the protein enrichment efficiency could be reduced when the starch granules and the protein bodies vary little in size and/or density, such as buckwheat (Tabtabaei et al. 2019; Zhu et al. 2021).

Several studies had showed that air classification can better maintain the native structure of globulin (Yang et al. 2021; Keivaninahr et al. 2021; Schutyser and van der Goot 2011). Globulins are the major storage proteins in pulses, canola, and oat. 11S and 7S are the two major plant globulin families. Plant globulins generally have a compact structure with hydrophobic groups hidden inside the globular structures while hydrophilic groups exposed to the aqueous environment (Mäkinen et al. 2016). Yang et al. had demonstrated that air-classified pea protein concentrate has relatively lower surface hydrophobicity than its counterparts extracted by conventional solvent methods. The fluorescence analysis showed that aromatic amino acids were less exposed to water phase in air-classified pea protein concentrate. Both results suggested that the native structure of pea globulins was preserved after air classification as compared to solvent extraction (Yang et al. 2021). A recent study demonstrated that the emulsions prepared by air-classified faba bean and pea protein concentrates appeared to have smaller particle size at pH 7 (Keivaninahr et al. 2021). The better solubility and less aggregation might allow the air-classified faba bean and pea protein to better diffuse and adsorb at the water/oil interface and stabilize the oil droplets (Keivaninahr et al. 2021). The other components present in the protein-enriched fraction can also contribute to the product mechanical structure, nutritive value and sensory properties. For example, the existence of 1% of oat β-glucan not only provided health benefit to the high-quality gluten-free bread but also increase loaf volume and crumb structure of high-quality gluten-free bread by contributing to the viscous property of the dough (Lazaridou et al. 2007). On one hand, starch and fiber may impair protein functionalities. For example, the gelling capacity and gel

strength of air-classified pea protein (55% protein content) were significantly lower than pea protein isolates (>85% protein content) by solvent extraction in wet methods. The starch and fiber hindered the protein-protein interaction development, and their presence resulted in coarse network structure and large pore size, thus leading to gel with significantly reduced mechanical strength (Yang et al. 2021).

Advantages, including the low consumption of water, no requirement of stabilization process after separation and readily scalable for potential applications, allow air-classification to be one of the most popular techniques to obtain plant proteins from pulses and cereals.

13.2.2 Electrostatic Separation

Electrostatic separation is another dry fractionation technique that may be applied to separate starch granules and protein bodies of similar size and/or density, thus can be complementary to air-classification. Based on the different triboelectric properties of different plant components, the electrostatic separation method generates finely milled flour particles with different charges, polarities, and magnitudes, and then these particles are separated when exposed to an electric filed (Zhu et al. 2021). Based on the methods used to impart surface charge, there are three types of electrostatic separators, induction charging, corona discharge, and tribocharging. Induction charging and corona discharge separators are more commonly used to separate minerals and mixed plastics, which contain components of significantly different conductivities. Tribocharging separation more effectively separates plant particles with small size and similar electrical conductivity since tribocharging unit allows those components charged with different polarities of different magnitudes (Zhu et al. 2021). Tribo-electrostatic separation is conducted by pneumatically conveying the flours through charging tubes to impart a positive or negative charge to the surface of the protein and carbohydrate particles. The charge of particle depends on its tribo-charging behavior and the contact medium in the charging tubes. For example, after contacting with polytetrafluoroethylene (PTFE) in the charging tube, the navy bean protein particles gained a net positive charge due to the presence of ionizable functional groups while carbohydrate particles showed no charge and/or a weak negative charge due to their low ionizability (Tabtabaei et al. 2016). The electrostatic separation method has been studied to concentrate proteins from navy bean, lupine, soy bean, and oil seed cakes (Zhu et al. 2021). The purities of plant protein obtained by electrostatic separation are comparable to those obtained by air-classification, leading to protein flours with protein content of 44% for rapeseed oil cakes and 58% for lupin (Zhu et al. 2021).

The protein concentrates obtained from electrostatic separation possess a similar protein composition profile to that of the original flour, mainly albumin, legumin and vicilin, and the protein native structure and functionality can be well preserved. For example, Tabtabaei (Tabtabaei et al. 2019) analyzed the protein composition of navy bean protein flours obtained by electrostatic separation to that of original flour and protein isolates by solvent extraction. The SDS-PAGE results showed that the

electrostatic separated protein fraction exhibited a similar pattern to the original flour with a slightly higher tendency of collecting more vicilin. On the other hand, navy bean protein isolated from solvent extraction method eliminated water-soluble albumins, which may have been lost during isoelectric precipitation stage (Tabtabaei et al. 2019). Thus, electrostatic separated navy bean protein fraction possessed higher solubility at pH around the isoelectric point of navy bean globulins because of the collection of water-soluble albumins (Tabtabaei et al. 2019).

Jafari et al. (Jafari et al. 2016) employed circular dichroism and fluorescence spectroscopy to examine the secondary structure and conformation of navy bean proteins obtained by electrostatic separation. The result demonstrated that no significant protein structural alteration was observed before and after electrostatic separation. The preservation of protein native structure may explain the observation that the electrostatic separated navy bean protein fraction surpassed its counterpart obtained by solvent extraction in solubility, emulsion stability, foaming expansion, and foam volume stability (Tabtabaei et al. 2019).

The processing parameters of electrostatic separation also impacted protein functional properties. For instance, the protein fraction collected from the top and middle part of the electrode plate had slightly higher protein fractions (37%) than that collected from the bottom (35%). The higher protein fraction (collected from top and middle part of the electrode plate) contained more small protein particles and small size starch fragments, which contribute to high water and oil absorption capacity (Tabtabaei et al. 2019; Pelgrom et al. 2015).

Beside the protein composition profile, structure, and functionality, electrostatic separation may also impact the sensory properties of the protein-enriched fractions. For example, Basset et al. found that lignin (negative charge) in the rapeseed oil cake can be separated from protein fraction (positive charge) through electrostatic separation. Thus, the lignin content decreased from 17% (dry mass) in the original rapeseed oil cake to 5% in the protein-enriched fraction. This resulted in protein fraction with lighter and brighter color, which makes it more desirable in food products by consumers (Basset et al. 2016).

Dry separation methods, such as air classification and electrostatic separation, have attracted great attention from industry because they use no water, protein separation by a physical approach that is scalable and allows preservation of protein structure and other valuable components. The high nutritive value and good functional properties of the protein enriched fractions allow them to be widely used in food products such as bakery, pasta, meat analogs, and dairy-free products. For example, the potential of using air-classified protein fraction in layer cake and sponge cake (Gómez et al. 2012), in pasta (Duta et al. 2019) and bread (Lazaridou et al. 2007) have been studied. However, the relatively low protein purity and the existence of other components, such as starch, polyphenols, lipids and dietary fibers, may have adverse influence on the protein functionalities and sensory properties required in food formulations. For example, the emulsifying properties of pea protein concentrate obtained by dry fractionation were significantly lower than those of pea protein isolates obtained by solvent extraction, which was attributed to the high portion of non-protein components (Karaca et al. 2011; Saldanha do

Carmo et al. 2020). On the other hand, in food formulations where protein functionality is not highly required, incorporation of protein flours will further increase dietary fiber in the final food products for improved nutritive quality and additional health benefits (Han et al. 2010). This also reduces the by-product streams after protein extraction and makes better use of valuable components in cereals and pulses. Examples of the use of protein enriched fractions include liquid food products, such as plant-based beverages and protein shakes, and solid and semi-solid food products, such as soups, meat analogs, bakery, pasta, and texturized products.

There are still research gaps and challenges that need substantial research efforts with the aim to improve separation efficiency and scale up the process. Both techniques involving dry milling, the exposure of enzymes such as lipase and lipoxygenase to air may trigger the oxidation reaction of lipid, generating unpleasant flavors. Thus, treatments before or after air classification to eliminate off-flavors are needed for some plant sources to obtain protein-enriched fraction with premium sensory properties. For electrostatic separation, the understanding on the internal bonding between components and the tribocharging properties of various raw materials is insufficient. Phytates, phenolic compounds, and enzyme inhibitors may be concentrated in the protein-enriched fraction after electrostatic separation, which may impact the nutritive value, digestibility, and functionality in different ways (Laguna et al. 2018; Saldanha do Carmo et al. 2020). More studies are required to refine the electrostatic separation process to eliminate/enrich those components from the protein fraction. More recently alternative forces fields such as magnetic field have been used together with triboelectric separation in mineral and plastic industries to increase separation efficiency, which may have the potential to be applied for plant protein separation in food industry (Zhu et al. 2021). Furthermore, the current research on dry separation methods mainly focuses on optimizing the process for improved protein recovery and purity. More research to characterize protein structures and functional properties are required to better understand the potential and challenges of using those protein fractions in food formulations as functional ingredients in the future.

13.3 Wet Processes

13.3.1 Solvent Extraction

Solvent extraction is widely used to obtain protein concentrates and isolates from plant sources. The solvent extraction of protein is usually divided into two steps. Proteins are firstly dissolved in solvent, then separated from other components to achieve high protein purity. Depending on the sources, some pre-treatments, such as cleaning, sorting, milling, defatting, and heating, may be needed before solvent extraction (Kumar et al. 2021b).

The efficiency of the protein isolation method depends on the nature of the protein sources. For example, the integrity and thickness of the cell walls could be an

obstacle for protein extraction from some plant sources. Cell wall disruption before solvent extraction, either by enzymatic method or physical techniques, accelerate the mass transfer, accessibility of solvent and extraction kinetics (Jung and Mahfuz 2009). Thus, it has been suggested by many studies that using novel technologies concurrently with solvent extraction allows higher protein yields (Kumar et al. 2021b). The novel technologies applied for raw materials pre-treatment and/or coupled methods to wet extraction include subcritical water extraction, enzymes, high pressure, pulsed-electrical field and high voltage electrical discharge, microwave, vacuum, and ultrasound assisted extraction. These techniques employed to assist cell lysis may involve heat, pressure, and mechanical forces, leading to protein structural changes at different levels. For example, (Gross and Jaenicke 1994) temperature is an extrinsic factor that can impact protein structure during solvent extraction. Heating of globular proteins in solvent above their denaturation temperature enhances ruptures of various intermolecular and intramolecular forces that stabilizing the native protein structure, leading to reversible and/or irreversible change to protein tertiary and secondary structures. The unfolded protein molecules may associate with each other through intermolecular interactions to form soluble and/or insoluble aggregates, leading to possible precipitation and changes on the protein functionalities. The electric fields used in pulsed electric field and high voltage electrical discharge technologies may also bring alterations to protein structures. During electric field extraction, the protein polar groups can absorb energy to generate free radicals which may interrupt forces that stabilize protein structure, including Van der Waals forces, hydrogen bonding, hydrophobic interaction, and disulfide bonds. The stronger the electric field and longer time of treatment, the higher degree of protein denaturation and higher potential to form protein aggregation (Li 2012).

Consequently, the proteins extracted through these solvent extraction methods are likely to possess different functionalities. Thus, it is necessary for the industry to understand the impacts of various extraction techniques on the protein structure and functional properties that directly determine their applications. Therefore, the impacts of different extraction technologies on protein structure and functional properties will be reviewed in detail in this chapter. The advantages and challenges of using these technologies to extract plant proteins will also be addressed.

Moreover, the protein composition of the extracts is dependent on the solvent used and the extraction processing parameters. For example, in pulses (peas, beans, etc.), and some cereals (oat, rice, etc.), the major storage proteins are albumins and globulins. These two types of proteins showed different solubility and functional properties. Albumins have high solubility at a wide range of pH while globulins have low solubility at pH around their isoelectric points (pI 4–5). Thus, different solvent extraction methods lead to extracts with different albumin/globulin ratios (Yang et al. 2021). For example, the isoelectric precipitation method could hardly recovery albumins since albumins have high solubility at the isoelectric point of globulin. Albumins are eliminated when the globulins are collected as a precipitant at their pI (Yang et al. 2021). In addition, the nutritive values are different between albumins and globulins. For example, pea albumins have higher lysine, valine, tryptophan,

threonine, methionine, and histidine than pea globulins. From this aspect, the extraction methods that remove albumins may reduce the nutritive value of the pea protein as a functional ingredient. Therefore, the influences of extraction methods on protein composition will be also addressed in this chapter.

13.3.1.1 Alkaline and Salt Solution Extraction

Alkaline and salt solution are two of the most conventional and widely used approaches to extracting protein from plant resources. For alkaline extraction, proteins are dissolved from the flour under alkaline conditions (pH 8–11). Alkaline treatment works efficiently for protein extraction through two mechanisms. Firstly, alkali disrupts the cell wall through partial removal of lignin and changing the structure of the polysaccharides on the cell wall (Perović et al. 2020). Secondly, proteins become more soluble under alkaline conditions since neutral and acidic amino acids will be ionized at alkaline pH. Moreover, the use of alkali could break the disulfide bonds leading to increased protein solubility (Sari et al. 2015). The albumin and globulin protein fractions from plant sources can also be extracted by salt solutions (NaCl, KCl, K_2SO_4, etc.), especially the globulin proteins have high solubility in salt solutions.

After dissolving in alkaline or salt solutions, proteins are separated from other components in the flours (lipid, starch, pectin, soluble fibers and insoluble fibers, etc.) through several techniques, such as isoelectric precipitation and micellar precipitation. For the isoelectric precipitation method, the pH of the solvent is adjusted to the pI of globulins where they have the lowest solubility. Then the globulins are collected as precipitant. In the micellar precipitation methods, protein molecules form micelles at low ionic strength and solubility decreases. The protein micelles are then collected. The protein ingredient isolated by alkaline and salt solution can reach relatively high purity and yield than dry separation methods, such as sieving and air classification (Momen et al. 2021). Alkaline-isoelectric precipitation method has been widely used to prepare protein isolates from plant sources, such as pulses, cereal, and oilseeds (Momen et al. 2021). The protein contents of isolates prepared by isoelectric precipitation from pulses, such as chickpea, faba bean, lentil, and pea range from 82 to 89% (Karaca et al. 2011). Tanger et al. had reported that 50% of the protein in parent pea flour can be recovered by alkaline-isoelectric precipitation and the protein content was 75%. Salt solution-micellar precipitation method can reach similar protein content (75%) but a relatively lower yield (25%) (Tanger et al. 2020). Depending on the pea varieties, Stone et al. found that 60–75% of the protein from the flour could be extracted by alkaline-isoelectric precipitation and salt solution-dialysis methods while only about 30% by salt solution-micellar precipitation methods (Stone et al. 2015).

Separation methods used in conjunction with the alkaline/salt solution influence protein composition, structure, and functionality from many levels. The alkaline and salt solutions followed by separation methods may selectively isolate some protein components which in turn influence the protein composition and functionality. For example, Yang et al. found that the pea protein extracted by alkaline solution tended to have a higher amount of 11S (legumin) while those extracted by salt solution have

more 7S (vicilin and covicilin). This may be attributed to the slightly lower solubility of 11S in diluted salt solution than 7S. The different 11S/7S ratio contributed to the different functionalities of pea proteins, especially in gelation (Yang et al. 2021). It had been reported that 11S legumin can form randomly constructed insoluble large aggregates through disulfide bonds and noncovalent interactions upon heating which impair the formation of protein network, resulting in poor gelling capacity (Mession et al. 2015; O'kane et al. 2005).

Moreover, the alkaline solubilization-isoelectric precipitation eliminates albumins during protein extraction since albumins have high solubility at pH 4.5 (the pI of globulin). The salt solution-micellar precipitation excluded albumins since albumins are not involved in the micelles formation in diluted salt solution (Yang et al. 2021; Tanger et al. 2020; Hadnađev et al. 2018). Tanger et al. stated that the pea protein extracted by salt solution-dialysis had higher solubility than those extracted by isoelectric and micellar precipitation methods, which were attributed to the coexistence of albumins after dialysis (Tanger et al. 2020).

In some cases, increasing alkaline conditions could result in higher protein yield and protein content (Akbari and Wu 2015), but the exposure of proteins to extreme pH (>10) can trigger protein partial unfolding (Ruiz et al. 2016; Jiang et al. 2009). The forces that stabilize protein structure may also be partially destroyed by high pH, resulting in the alteration of the protein structures. Protein structures are maintained by various covalent and noncovalent interactions, such as disulfide bonds, hydrophobic interaction, electrostatic and van der Waals interactions, between multiple groups in the proteins. For globulins, the tertiary structure is important to the relocation of the nonpolar residues at the core and the exposure of the hydrophilic residues at the exterior of the protein molecule. The changes in the tertiary structure inevitably alter the surface hydrophobicity of the protein, leading to variation in their functionalities. For example, by measuring the intrinsic fluorescence of pea proteins extracted by different solvent methods, Yang et al. found that alkaline solution (pH 9, 2 h, 22 °C) had a stronger impact on protein tertiary structure than salt solution, resulting in a higher surface hydrophobicity because of the exposure of the hydrophobic core to the exterior aqueous solution (Yang et al. 2021). Jiang et al. also showed that soy protein isolated by high alkaline conditions (pH 10–12, 1–4 h, 20 °C) had a substantial increase in intrinsic tryptophan fluorescence intensity, indicating that high pH leads to the lost of some tertiary structure to expose the hydrophobic tryptophan residues to aqueous environment. The surface hydrophobicity plays an important role in protein functionalities, such as solubility, interfacial properties, oil holding capacity, and flavor binding capacity. The increased surface hydrophobicity allowed soy protein to approach the oil–water interface faster, resulting in improved emulsifying properties (Jiang et al. 2009). For alkaline extracted quinoa protein, the loss of tertiary structure exposed more interior groups, which facilitated protein–protein interactions at the oil–water and air–water interface to form protein network and contributed to more stable emulsions or foams (López-Castejón et al. 2020).

The secondary structure of protein is stabilized by intrachain hydrogen bonding between the N-H group and the C=O group. Depending on the conditions used in the extraction, alkali and salt have the possibility of dissociating the hydrogen bonds

thus altering the secondary structure of protein. For globulins, the irreversible denaturation resulted in a molten globule-like conformation status. Jarpa et al. reported that the higher the extraction pH, the higher degree of unfolding happened on lentil proteins as analyzed by Fourier transform infrared spectroscopy (Jarpa-Parra et al. 2014). This is further supported by differential scanning calorimeter analysis of the denaturation temperature and the denaturation enthalpy (ΔH) of chia proteins extracted by alkaline solubilization-isoelectric precipitation (pH 10). The results showed that the protein denaturation temperature decreased from 116 °C in the chia flour to 108 °C after extracted by alkaline-isoelectric precipitation method while ΔH decreased from 151 to 109 J/g after extraction (Coelho and Salas-Mellado 2018). Ruiz found that the ΔH of quinoa protein significantly decreased from 10.2 J/g to zero when the extraction pH increased from 8 to 11 (Ruiz et al. 2016). Thus, the higher the extraction pH, the stronger impact on the protein secondary structure. The partial denaturation of lentil protein was beneficial to its gelling properties. The proteins extracted at pH higher than 9 formed gels with higher mechanical properties than those extracted at pH 8, since the partial unfolding of lentil protein extracted at pH 9 and 10 resulted in higher degree of denaturation upon heating and more exposure of hydrophobic groups that buried in the hydrophobic core. More active groups promoted the development of protein–protein interaction, which facilitated the protein network formation (Jarpa-Parra et al. 2014). For comparison, salt solution-micellar precipitation method resulted in pea protein with higher ΔH and denaturation temperature than that from alkaline solubilization-isoelectric precipitation method which indicated that micellar precipitation preserved more native pea protein.

The partial unfolding of protein leads to the exposure of the interior core to the aqueous environment, which potentially triggered protein aggregations through various intermolecular interactions such as intermolecular hydrogen bonding, hydrophobic interactions, and disulfide bonds. Depending on the case, protein aggregation may impact protein functionality positively or negatively (Momen et al. 2021). During alkaline or salt extraction, protein composition and structure changes concurrently occur with the formation of protein aggregation. Thus, it is hard to attribute certain functionality changes solely to the formation of protein aggregation. For example, Yang et al. have found that pea protein extracted by salt solution-dialysis method formed aggregates through intermolecular β-sheets, which curtailed its solubility and thermal-induced gelling capacity (Yang et al. 2021). The aggregates hindered the unfolding of protein molecular during heating. Thus, the salt solution-dialyzed pea protein formed condensed particulate gel through aggregates interaction with a limited amount of junction zones, resulting in significantly lower gel mechanical strength as compared to those made by pea protein extracted by ultrafiltration or micellar precipitation (Yang et al. 2021). Jiang et al. reported that soy protein isolated through alkaline solubilization-isoelectric precipitation formed disulfide-mediated aggregates, which decreased the protein solubility. However, due to the increase in surface hydrophobicity, the overall soy protein emulsifying activity and emulsion stability remarkably improved (Jiang et al. 2009).

A suitable level of protein structure changes can increase the protein surface hydrophobicity, which may be beneficial to functional properties like foaming, emulsifying, flavor binding, and oil holding capacity. However, severe denaturation during extraction should be avoided because the extensive unfolding of protein may result in protein-protein interaction randomly, leading to the formation of insoluble aggregates. Though the impacts of extraction methods on aggregation formation have been proven by many studies, controlling the protein aggregation degree through manipulating the extraction conditions has not been systematically explored. The impact of aggregation on protein functionality properties has not been studied in detail yet. Thus, more research is needed to understand the impact of solvents on protein structure and explore how to manipulate protein structure and its thermal stability through different extraction conditions. This knowledge will allow strategies to produce high-quality plant protein adapting to various food applications.

Besides the above-mentioned effects, alkaline and salt solutions may also induce other protein structure alterations, such as hydrolysis, deamidation, Maillard reactions, and covalent crosslinking via cysteine, dehydroalanine and lysine residues, which have been summarized in many previous review papers, thus are not included in this chapter (Deleu et al. 2019).

Alkaline and salt solution extraction methods, especially alkaline solution-isoelectric precipitation, have been applied to a wide range of plant sources to achieve protein isolates with relatively high yield and high protein purity on a large scale. However, these conventional extraction techniques generate a large amount of water waste with alkaline or salts. Though increasing the alkaline pH can improve extraction efficiency, high pH has a negative impact on the sensory properties of protein isolates, such as dark color and unpleasant flavor, which may lower the consumer acceptability and hinder their application in food products. Additionally, essential amino acids lysine and cysteine residues are not stable in high alkaline condition (pH >9) (Chang et al. 1999). Thus, a balancing point between protein extraction efficiency and protein quality (functionality, sensory property, nutritional value, etc.) is needed to be found and maintained for each kind of plant source. Recent research on new technologies has shed light on the possibility of using alkaline and salt solution extraction methods concurrently with novel technologies to produce high-quality protein ingredients with high efficiency under milder condition.

13.3.1.2 Emerging Solvent extraction Technologies

In recent years, novel solvent extraction technologies have emerged and gained popularity for extracting proteins of industrial interest. Aqueous two-phase system (ATPS) is one of these emerging solvent extraction methods (Fig. 13.2). The ATPS can be usually formed when a water-soluble polymer and another polymer or certain inorganic salts are mixed above critical concentration, where the 2 phase components are separated into two clear aqueous phases. Conventional ATPSs are formed by two incompatible polymers, i.e., dextran and polyethylene glycol (PEG), or a polymer (usually PEG) and a salt, in an aqueous solution (Lee et al. 2017). The

ATPS has been applied as a superior method for protein purification. This superiority is owing to the high water content and low interfacial tension in this phase separation system, which protects the proteins and prevents their denaturation or loss of biological activity (Xu et al. 2015). Also, protein extraction using ATPSs is considered to be environmentally friendly because traditional volatile solvents are not used in ATPS process (Han et al. 2011).

Ionic liquids (ILs) are substances that are composed of cations (e.g. 1,3-dialkylimidazolium or N-alkylpyridinium cation) and weakly coordinating anions (Sheldon et al. 2002). ILs have many promising properties. Unlike organic solvents, ILs are generally non-flammable and have negligible vapor pressures, thus do not evaporate in normal conditions. Also, the physicochemical and biological properties of ILs can be tuned or tailored by the manipulation of the cationic and anionic constituents. ILs can be easily recycled. Thus, ILs could contribute significantly to the development of green technologies for protein extraction by (1) replacing toxic and flammable organic solvents; (2) reducing chemical waste and pollution; (3) increasing the chemical process safety (Ding et al. 2014; Freemantle 2010). These remarkable properties make ILs superior solvents used in the ATPS for the purification of biological products, including protein extraction. In 2007, Du et al. initiated the first study of protein extraction using the IL-ATPS and successfully extracted proteins from human urine into the IL-rich phase (Du et al. 2020). Ding et al. developed the extraction process based on guanidinium ILs–ATPS, where functional guanidinium ILs and phosphate solution were designed for protein purification, including lysozyme, trypsin, ovalbumin, and bovine serum albumin (BSA). This method exhibited high purification efficiency (over 90%) for all protein samples, and the purity of lysozyme reached near 100%. ILs–ATPS based on 1,1,3,3-tetramethylguandine acrylate guanidine ionic liquid (TMGA) was applied for the extraction of proteins (Zeng et al. 2013). The extraction efficiency was reported up to 99.62%. Such high extraction efficiency was achieved because ILs can form aggregates above the critical aggregation concentration of TMGA, which then promote more BSA to be enveloped by IL aggregates.

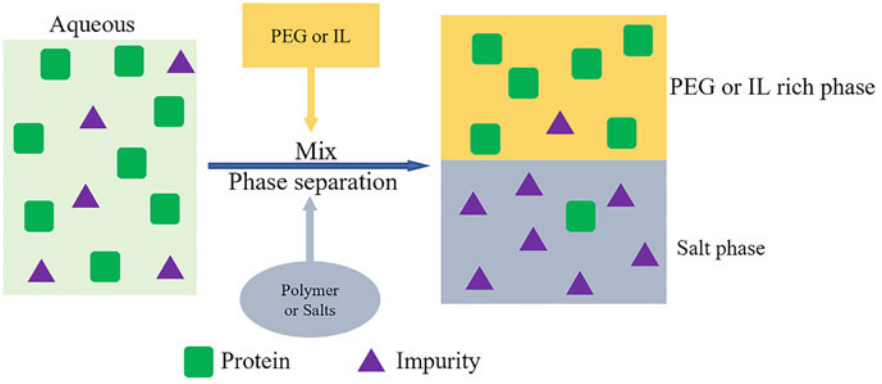

Fig. 13.2 The extraction process of ATPS

The changes of the protein secondary structure by IL-ATPS process were investigated using UV–vis spectrophotometer, FTIR and circular dichroism (CD) spectrum. The results indicated that the treatment of ILs–ATPS did not change the secondary structure of the extracted proteins, and there were no chemical bonds between the proteins and ILs. In a study carried out by Zeng et al., UV–vis and FT-IR spectra suggested that no chemical interactions were found in the extraction process of a TMGA/K_2HPO_4 aqueous two-phase system (Zeng et al. 2013). Besides, the authors suggested that hydrophobic interaction, hydrogen bonding and the salting out effect performed critical roles in the extraction process. For example, the top K_2HPO_4 phase has a strong salting-out effect and its presence in the IL-ATPS system drives proteins to transfer into the IL-rich top phase through hydrophobic interactions.

Nevertheless, developing ILs–ATPSs as a protein extraction method for large-scale industrial use has some challenges. The first major concern is the cost of the materials for IL formations. Laboratory-scale ILs are 5–20 times more expensive than organic solvents. However, ammonium and choline-based ILs are reported to be commercially available at a reasonable price (Lee et al. 2017). Besides, the selection of ILs should also be made concerning their biocompatibility with proteins, toxicity, and biodegradability. It has been reported that Ammonium-based ILs made of 2-hydroxyethyl-N, N, N-trimethylammonium (known as the cholinium cation) and Carboxylic acid ILs possess outstanding biodegradability and marginal toxicity. To apply IL-ATPS technology for protein extraction from plant sources, knowledge about the partition behavior of the target plant protein is still required and the effects of ILs-ATP extraction on the plant protein structural and functional properties should be further investigated in future works.

Over the past decade, deep eutectic solvent (DES) has been applied for protein extractions based on ATPS. DES is a eutectic mixture consisting of a hydrogen-bonding acceptor with a hydrogen-bonding donor (e.g., amines, alcohols, and acids) (Xu et al. 2015). DES-ATPS is non-toxic and has better biodegradability and lower cost, thus such extraction technology is considered as a substitute for its ILs counterpart. In a study carried out by Xu et al., four kinds of DES combining choline chloride with different hydrogen bond donors (ethylene glycol, glycerol, D-glucose, D-sorbitol solution) were generated under heating at 80 °C (Xu et al. 2015). The DES-based ATPS systems were made by mixing the DES with K_2HPO_4 solution for BSA extraction. The results showed that as high as 98% of the BSA could be extracted into the DES-rich phase in a single-step extraction. In another study carried out by Li et al., 99.8% of extraction efficiency for BSA could be achieved using Be–U (1.2 g)/K_2HPO_4 (2.0 mL) ATPS using a facile method similar as the above DES-based ATPS method (Li et al. 2016b). Conformation of BSA was not changed during the extraction process based on UV–vis spectra, FTIR, and CD spectra measurements (Xu et al. 2015). To date, most of the studies about DES-based ATPS method are related to biomedical applications for separations of peptides, DNA and RNA. Extracting plant-based protein using such method has not been reported. As an emerging ATPS method that has relatively lower cost and easier processing method compared to IL-based ATPS, the potential of applying

DES-based ATPS method to extract plant-based proteins should be explored and its effect on protein structures and functionalities needs to be studied in the future.

Subcritical solvent extraction is another emerging solvent extraction method where the extraction solvent is kept in the liquid phase under high temperature and pressure. Water is the most used subcritical fluid in extraction processes, which uses hot water between 100 °C and 374 °C under high pressure to maintain the fluid state (Kataoka et al. 2008). The key feature of subcritical solvent extraction is that the relative dielectric constant of water decreases with increasing temperature. Water is a very polar solvent at room temperature, with a dielectric constant of around 80. While the dielectric constant significantly decreases to 27 when water is heated up to 250 °C, which is similar to that of ethanol. Therefore, subcritical water has the ability to dissolve hydrophobic substances to be used for the extraction of hydrophobic substances from agricultural products. In a study by Lu et al., enzyme-assisted SubH_2O treatment successfully improved protein extraction from heat-denatured soy meals (Lu et al. 2016). When compared to the low protein yield (16.4%) extracted by conventional alkaline extraction and acid precipitation at 25 °C, subcritical water significantly increased protein yield to around 60%. This increased protein yield can be attributed to the fact that the more hydrophobic proteins can be dissolved under the low dielectric constant of subcritical water conditions. Consequently, the obtained soy protein isolate extracted by subcritical water extraction process contained a higher amount of hydrophobic amino acids when compared to untreated soy proteins. Intrinsic fluorescence test indicated a partial unfolding of the extracted proteins upon SubH2O treatment, which could then contribute to a more flexible protein conformation. In this case, more hydrophobic patches were exposed outside to enhance the surface hydrophobicity of the extracted proteins, which significantly enhanced their interfacial properties. SubH2O treated proteins showed a significantly higher protein surface loading at the oil–water interface of over 5 mg/m^2, twofolds higher than that of non-treated proteins (3 mg/m^2). As a result, the emulsion droplets stabilized by SubH2O treated proteins showed a narrow size distribution after 20 days of storage when compared to those stabilized by non-treated proteins.

These findings suggest that subcritical solvent extraction, as an emerging solvent extraction method, is of great potential to extract plant-based proteins at an industrial scale and enhance the structural and functional properties of the extracted proteins. Future research is required to focus on minimizing the effect of SubH2O on protein conformational changes. Ho et al. recommended lower temperatures (<160 °C) when applying SubH2O to treat proteins, since proteins are vulnerable to thermal denaturation (Ho et al. 2007). Also, developing SubH2O-based extraction method with higher cost efficiency is required for industrial applications.

13.3.1.3 Reverse Micelle Extraction/Microemulsion

Reverse micelle extraction (RME) is a relatively new liquid–liquid extraction technology and especially for protein isolation and purification. Reversed micelle, also known as water-in-oil microemulsions, is thermodynamically stable and self-assembled nanometer-sized isotropy particle that is formed by the aggregation of

surfactant molecules in organic solvents, consisting of a polar inner core and an inner layer made of the surfactant hydrophilic head (Bu et al. 2014). The general mechanism of RME to extract plant proteins consists of two fundamental steps: forward extraction and backward extraction. Forward extraction is to solubilize proteins into inner aqueous cores of reverse micelles, while backward extraction is a process where the solubilized protein is transferred into an aqueous phase to be recovered, also resulting in separation of the aqueous phase and oil phase (Sun and Bandara 2019).

In recent years, the application of RME to extract plant-based proteins has become an area of considerable interest. As a promising alternative of alkaline extraction and isoelectric precipitation method for protein extraction, RME technique has comparable extraction efficiency to the traditional extraction method with good preservation of protein native structures. Protein conformational changes after RME process and their effects on the protein physicochemical properties and functionalities have been studied. Zhu et al. investigated the secondary structures and functional properties of defatted wheat germ protein extracted by sulphosuccinic acid bis (2-ethylhexyl) ester sodium salt-based RME (Zhu et al. 2010). When compared to control proteins which were extracted by alkaline extraction and isoelectric precipitation, RME extracted proteins showed less random coil and more α-helix. Amino acid analysis suggested that RME extracted wheat germ protein containing high levels of threonine, histidine, alanine, arginine, glycine, serine, cysteine, proline, and lysine, while traditional alkaline extraction treated protein show a lower number of these amino acid residues owing to their destruction by alkaline treatments. The lysine content of RME-treated wheat protein (6.93 g/100 g protein) was dramatically higher than that of alkaline extraction treated protein (4.96 g/100 g protein). As lysine is considered as the first limiting essential amino acid in cereal proteins, 6.93 g/100 g protein of lysine in RME-treated wheat germ proteins is higher than the reference pattern for infants (5.80 g/100 g protein) issued by the FAO/WHO/UNO. Moreover, RME extracted proteins showed significantly higher water solubility than that of the control group at all pH values ($p < 0.05$). In a later study carried out by Bu et al., sodium bis(2-ethylhexyl) sulphosuccinate (AOT)-based reverse micelle system and AOT/Tween 85-based reverse micelle system were used to extract protein and oil from soya bean flour (Bu et al. 2014). Scanning electron microscopy analysis showed a significant microstructure difference between SPI obtained by alkali solution and acidic precipitation method and AOT reserve micelle extraction, where the former exhibited a roughly spherical shape, and the latter revealed a mostly lamellar structure. Fourier transform infrared spectroscopy (FTIR) revealed that SPI extracted using RME had no unordered structure when compared to that from an aqueous solution. This was consistent with the previous study we discussed above that RME technology had the least influence on the secondary structures of proteins and could remain the native conformation of the proteins. In addition, oil products from soya bean flour extracted by RME also showed lower acid and peroxide values. The surface properties of protein are related to various functional properties such as wetting, dispersibility, oxidative stability, flowability, and rehydration properties (Gaiani et al. 2010). Liu

et al. utilized X-ray photoelectron spectroscopy (XPS) to investigate the surface properties of walnut protein extracted by AOT-based RME (Liu et al. 2014). When compared to the surface of walnut protein extracted by NaOH solution (pH 9.0) at ratio 1:15 (w/v), the O atomic percentage on the surface of walnut protein powder extracted by AOT-based RME was higher, while the C and N atomic percentages were lower, indicating that the RME process could affect the original C, O and N components on the surface of walnut protein powder. This phenomenon was possibly owing to the oxidation of walnut protein surface during the RME process, which is related to functional properties of the protein powders such as wetting, dispersibility, oxidative stability, and rehydration properties (Gaiani et al. 2010).

On the other hand, some recent research reported that the RME process could improve the functional and/or nutritional properties of the extracted plant proteins. Du et al. reported that the RME process could efficiently modify the physicochemical properties of the extracted soybean 7S globulin, including decreased surface hydrophobicity and lower denaturation temperature(Du et al. 2020). Therefore, the gelling capacity of soybean 7S globulin extracted by AOT/isooctane-based RME was improved when compared to those extracted by traditional alkali extraction-acid precipitation method (Du et al. 2020) with a reduced gelling temperature of 82.6 °C, compare to 80.3 °C of alkali extraction-acid precipitation prepared proteins. And the gels are more elastic with increased storage modulus (G'). A study carried out by Zhao et al. indicated that soy proteins extracted by RME process exhibited an improved protein solubility index owing to lower degree of denaturation, which then further improved the foaming and emulsifying capacity (Zhao et al. 2018). Moreover, better nutritional characteristics were observed in total essential amino acids, amino acids score, and biological value when compared with proteins separated by alkaline extraction and isoelectric precipitation (Zhao et al. 2018). This can be explained by the fact that alkaline treatments could lead to chemical modifications of some essential amino acid residues, such as threonine and lysine residues.

These findings are of great value for better understanding the relationships between the RME process and the changes in the structural and functional properties of plant-based proteins, and for exploring the potential of applying RME technology in the food industry. However, future research is required to expand application areas of RME in food science. Firstly, current studies did not well explain the underlying mechanisms on how RME treatments could modify or improve the structures and properties of proteins. Most of the RME systems applied in plant protein extraction consisted of AOT and isooctane which are both not approved in food and drug applications. Thus, exploring more biocompatible and edible surfactants (e.g., Tweens and sugar esters) and solvents to build effective RME systems is essential in food or drug applications. Protein extraction using the REM method is also more expensive compared to traditional alkaline extraction and isoelectric precipitation (Zhao et al. 2018). Thus, the development of an REM method with higher cost efficiency is also required.

13.3.2 Energy-Assisted Extraction Methods

Conventional solvent extraction methods have been widespread applied in industry in recent decades due to their easy operation and simple instrumentation required. However, these conventional extraction methods are considered as inefficient due to the long processing time, high solvent and energy consumption, and some other disadvantages on the overall quality and acceptability of extracted protein, such as the disintegration of the structure of lysine and cysteine amino acid during alkaline extraction at high pH (Chua 2013). Thus, the research and development of emerging extraction technologies is now spreading and gradually being applied in the field of food industry. Although these techniques are introduced in recent review papers (Kumar et al. 2021a; Pojić et al. 2018), the systematic review on the impact on emerging extraction technologies on protein quality is still limited. Thus, in the following section, the protein structure and functional properties as impacted by energy-assisted extraction methods are discussed.

13.3.2.1 High Pressure

High-pressure processing is a non-thermal food processing technique and is gaining popularity as an alternative to conventional solvent-based extraction methods. The extraction is performed at ambient temperature, and avoids thermal degradation and loss of bioactivity of the extracted compounds. There is a differential pressure between plant cell interior and surrounding as the pressure increases, which leads to cell deformation and cell wall damage, and then the solvent penetrates into the cell to dissolve the bioactive components. In the pressure maintaining stage, the solvent continues to penetrate through the cell wall and dissolve the components. During the pressure releasing, the cell expands due to the pressure decrease in the cell to ambient pressure, which causes cell deformation to increase the diffusion of the compound. Therefore, high pressure-assisted protein extraction can result in high extraction efficiency during pressure increasing, maintaining, and releasing.

Protein subunits can be first dissociated under pressure. Then protein chain gradually unfolded with rising pressure, accompanied by some changes in the tertiary structures of proteins, such as exposure of more hydrophobic groups. The secondary structure can be changed at higher pressures, generally over 400 MPa and result in non-reversible denaturation. However, changes in the primary structure of proteins did not take place at a pressure below 2 GPa (Winter et al. 2007). Protein denaturation caused by high-pressure process is generally dependent on protein original structure, protein concentration, and pressure conditions. Generally, high-pressure processing would be combined with other extraction methods to extract protein. For instance, high pressure (200 and 500 MPa for 15 min) as a pre-treatment was applied to the full-fat soybean flakes to extract the protein during aqueous extraction processing respectively (Jung and Mahfuz 2009). The results show that high pressure of 200 MPa had a positive effect on protein extractability, increasing protein recovery to 81.5% from 73.5%, while the pressure of 500 MPa decreased it to 65.6% from 73.5% due to protein insolubilisation (Jung and Mahfuz 2009). Next, isolated soy proteins were obtained by isoelectric precipitation, and protein sample

showed lower surface hydrophobicity compared to that without high-pressure pre-treatment due to rearrangements of proteins conformation by high-pressure treatment, resulting in an increase in the water holding capacity (Jung and Mahfuz 2009).

The pressure at different levels can affect the protein structures to different degrees and in turn influence protein functionalities. Solubility is a very important functional property that relates to most of other protein functional properties. But the effect of high pressure assisted extraction on solubility was contradictory in the literature. For example, solubility of sesame proteins (Achouri and Boye 2013) with pH 2 and 5 can be increased under 200–500 MPa due to the dissociation of aggregates, which promoted more protein-solvent interactions. The same results were reported in kidney bean protein (Al-Ruwaih et al. 2019). However, the solubility of isolated soy protein extracted from full-fat soybean flakes by high pressure at 200 and 500 MPa for 15 min prior to enzyme-assisted aqueous extraction was decreased from 84.7% of enzyme-assisted aqueous extraction alone to 81.0 and 43.1%, respectively (Jung and Mahfuz 2009). Similar to results obtained by Peyrano (Peyrano et al. 2016), a reduction in the solubility of cowpea protein after the 5 min high-pressure process at 200 and 400 MPa occurred, which was due to the formation of insoluble high molecular weight aggregates through the exposure of hydrophobic residues or the creation of disulfide bonds between proteins. However, at 600 MPa solubility was similar to that of untreated samples, suggesting that higher pressure leads to highly soluble aggregates (Peyrano et al. 2016). Jung et al. (Jung and Mahfuz 2009) found that high-pressure pre-treatment at 200 and 500 MPa increased the water holding capacity of isolated soy protein extracted from full-fat soybean flakes during enzyme-assisted aqueous extraction processing but decreased the ability of the isolated soybean protein to bind oil. These results were attributed to exposure of polar amino acids to a more polar environment by high pressure, facilitating the interactions between protein and the solvent. In addition, viscosity can be enhanced significantly after high-pressure treatment, which was observed for full-fat soybean flakes (Jung and Mahfuz 2009; Galazka et al. 2000). The reason might be associated with the progressively higher amounts of cell breakage by high pressure that promoted extraction of okara components (protein and fiber) and then causing a higher content of intercellular components in the dispersions. Also, particle-particle interactions and build-up of structure lead to increased viscosity, which can be attributed to a large number of smaller particles from a few numbers of larger particles after high-pressure treatment. Higher viscosity can improve the textural and other functional properties (e.g., emulsifying property and gelation), which has wide application in food products, such as thickener and stabilizer in ice cream, beverages, and bakery. Moreover, the smaller particles after high-pressure treatment could form a denser gel network, which indicated that high pressure could enhance gel strength of proteins from pea (Sim et al. 2019), soybean (Li et al. 2011), and peanut (He et al. 2014). The increase in the number of hydrophobic interactions and disulfide bonds resulted from high pressure also contributes to the gel strength of protein. Finally, emulsifying and foaming properties could be altered by high-pressure treatment. An increase in emulsifying activity of proteins was observed

after high-pressure treatment, such as lentil protein isolate treated by 300 MPa for 15 min (Ahmed et al. 2019), kidney bean protein isolates treated by 200–600 MPa for 15 min (Ahmed et al. 2018), and soy protein isolate treated by 400–600 MPa for 15 min (Molina et al. 2001). This occurs because high pressure could unfold and partial denature protein molecules, exposing hydrophobic groups and absorbing more protein moieties at the interface between oil and water. Similarly, foaming capacity of proteins could also be improved by high pressure due to the exposure of hydrophobic amino acid residues to rapidly form and adsorb viscoelastic films at the air–water interface (Li et al. 2011) (Al-Ruwaih et al. 2019). However, some research reported opposite results. For instance, the formation of pea protein aggregates at high pressure (200–600 MPa for 5 min) reduced the molecular flexibility, thus the ability to form strong membranes at the air–water interface was decreased, leading to low foaming capacity (Chao et al. 2018).

Overall, high pressure can dissociate the protein into subunits and then unfold their structure to different degrees by different pressure levels and other factors (such as time and temperature). Then unfolded protein associate to aggregates (soluble or insoluble). Generally, the insoluble aggregate was formed at lower pressure level (200 MPa), then this insoluble aggregate was transformed into soluble aggregate at a higher pressure level (600 MPa), like soy protein (Tang and Ma 2009). Thus, understanding the pressure level in relation to protein structure changes such as unfolding an aggregation will enable a better control of high-pressure parameters for desirable properties in food industry. Additionally, since high-pressure technology caused a structural modification in proteins, it is applicable in the alteration of protein allergenicity. For example, the allergenicity of soy protein isolate decreased 48.6% after high-pressure treatment under 300 MPa for 15 min (Li et al. 2012). A similar phenomenon was reported by Penas et al. (Peñas et al. 2006), who confirmed the decrease in soy-whey protein allergenicity after the combination of both enzymatic hydrolysis and high-pressure treatment at 100–300 MPa for 15 min due to the removal of the conformational antigenic epitopes of the allergens. Moreover, high-pressure extraction is done at ambient temperature, thus, heat-sensitive components and nutrients can be avoided thermal degradation. High pressure can induce changes in the volatile profile of food products. For instance, high-pressure treatment (200 and 400 MPa for 10 min) improved the flavor in cooked rice based on the increase in alcohols and ketones as well as the decrease in aldehydes, which are three key flavor compounds in rice (Deng et al. 2013). Therefore, high pressure is gaining popularity as an alternative to conventional extraction methods.

13.3.2.2 Pulsed Electric Field and High Voltage Electrical Discharge

Pulsed electric field and high voltage electrical discharge are two kinds of pulsed electric energy technologies that have emerged for food processing (Vorobiev and Lebovka 2016). They are commonly designated as non-thermal treatment, which avoids undesirable changes in biological material. Pulsed electric energy technology has attracted interest for protein extraction since the protein quality is minimally affected during processing (Kumar et al. 2021a). Pulsed electric field extraction involves treatment of plant material by a number of pulses with high electric field

intensity in the range of 10–80 kV/cm for different duration times in the range of microseconds to milliseconds (Kumar et al. 2021a). This processing induces electroporation of the cell membranes, enabling a release of intracellular material (Okolie et al. 2019). Thus, pulsed electric field allows higher protein extraction efficiency within a shorter period of time. High voltage electric discharge extraction has a high-speed development in recent years, which is reported to be more effective than pulsed electric field to extract proteins because the application of electrical breakdown leads to bubbles division and improves the treatment efficiency(Roselló-Soto et al. 2015). This method produces high energy between two electrodes from high voltage electricity that causes cellular disintegration of tissues to extract valuable bioactive compounds (Boussetta and Vorobiev 2014).

Application of pulsed electric field and high voltage electrical discharge as an assisted treatment extraction of proteins resulted in the improvement of protein yield, accompanied with the reduced time and temperature for, such as *A. platensis* strain (Jaeschke et al. 2019), Green large algae *Ulva ohnoi* (Prabhu et al. 2019), rapeseed (Barba et al. 2015; Yu et al. 2015), mushroom (Parniakov et al. 2014) and beer waste brewing yeasts (Liu et al. 2012). So far, the technologies are most used for algae, fungi, and yeast protein extraction. The applications to crops such as cereals and pulses are still limited. In addition, efforts to study the protein structure changes by electric field extraction as a pre-treatment to assist the protein extraction is still at early stage. Several papers (Li 2012, Zhao and Yang 2009, Ji et al. 2019, Zhang et al. 2021) showed that the electric fields could modify protein structures. The main mechanism of protein structure modification is related to the protein polar groups that can absorb energy to generate free radicals, which could then trigger protein unfolding and aggregation. Since various interactions could be disrupted by free radicals, including Van der Waals forces, electrostatic and hydrophobic interactions, H-bonding, disulfide bridges and salt bridges, different levels of protein structure could be altered. For example, the secondary structure in soy protein was significantly changed when the applied pulsed electric field treatment intensity was over 35 kV/cm (Liu et al. 2011). The tertiary structure of horseradish peroxidase was changes at 5–15 kV/cm, while the tertiary structure had greater changes once the applied electric field strength was more than 15 kV/cm (Zhong et al. 2005).

Electric field strength and duration are critical factors in altering protein structure. Zhao et al. (Zhao and Yang 2009) applied pulsed electric field for pepsin treatment at several electric field strength levels (25.2, 30.4, and 35.6 kV/cm) for 0–500 μs. Self-aggregation of pepsin was observed especially at a high-intensity electric field of 35.6 kV/cm. Because the protein was subjected to the external electric field, buried hydrophobic side chains of globular proteins were exposed, leading to form self-aggregation through hydrophobic interactions. Moreover, the tertiary structure of pepsin protein became less defined and loose after being treated at 35.6 kV/cm of pulsed electric field for 0–500 μs as demonstrated by the near and far-UV circular dichroism spectra, and the changes were significantly increased with treatment time prolonged (Zhao and Yang 2009). They also found a gradual disruption of β-sheet structure and the emergence of intensity characteristic of random coil regions of structure with the increase of pulsed electric field treatment time from 200 to 500 μs

at 35.6 kV/cm (Zhao and Yang 2009). The loss of α-helix of secondary structure was observed in other enzymes and plant proteins after pulsed electric field treatment, such as lysozyme (Zhao et al. 2007), horseradish peroxidase (Zhong et al. 2005), polyphenol oxidase (Zhong et al. 2007), and soybean (Li 2012).

High voltage electrical discharge introduces energy directly into an aqueous solution or surrounding air via a plasma passage developed by a high current or high voltage electrical discharge between two submersed electrodes (Boussetta and Vorobiev 2014). The high voltage pulse induces a very high local electric point, shock waves, and formation of O_3 are yielded, causing disrupt the cell walls to release proteins (Boussetta and Vorobiev 2014). Several types of discharges have been known such as glow discharge, dielectric barrier discharge, and corona discharge. Among them, the corona discharge has drawn more attention in food industry (Dalvi-Isfahan et al. 2016). Some of the active species generated by discharges, such as hydroxyl radicals, atomic oxygen, and ozone, can interact with the extracted compounds and change the molecule configuration. For example, the active groups of pea protein could react with the radicals to form aggregates via hydrophobic interactions and disulfide bonds (Zhang et al. 2021). Moreover, the second structure of wheat protein was influenced under applied voltage of 10–15 kV through changes in hydrogen-bonding pattern of wheat protein (Singh et al. 2015).

The structural changes influenced protein functional properties, depending on the electric field strength and duration. For example, the solubility of soybean protein isolates was increased with the increase in the pulsed electric field intensity and processing time at constant pulse width 2 μs, but declined when the pulsed electric field strength and treatment time were above 30 kV/cm and 288 μs. (Li et al. 2007). Because the interactions between protein molecules and water could be enhanced with a mild pulsed electric field strength or treatment time, leading to an increase in solubility. But a stronger treatment condition caused them to decrease due to dissociation, denaturation, and aggregation of the protein. The same trend was observed for protein ingredients by pulsed electric field-treated canola protein isolate (Zhang et al. 2017). Solubility of protein is closely related to functional properties such as emulsifying, foaming, and gelling properties. Zhang et al. (Zhang et al. 2017) found that the emulsifying capacity and emulsion stability increased 1.13 and 1.21 times with the voltage and treatment time of 30 kV and 180 s, respectively. The foaming capacity and foaming stability increased 1.33 and 1.49, and 1.4 and 1.51 times with the voltage and treatment time of 30 kV and 180 s, respectively (Zhang et al. 2017). Since pulsed electric field treatment could unfold the protein structure to let the exposure of more buried hydrophobic groups and regions. Proteins with increased surface hydrophobicity promoted interactions between protein and oil droplets to form a more elastic film at the air–water interface. However, overtreatment with higher voltage, longer treatment time, faster pulse frequency and wider pulse width caused the larger aggregations formed by noncovalent bonds, such as hydrophobic interactions, electrostatic interactions, and hydrogen bonds. This subsequently led to a slight decrease in the solubility and surface hydrophobicity and then negatively affect on emulsifying and foaming properties. In addition, the apparent viscosity of proteins can be affected by pulsed electric field treatment.

Xiang et al. (Xiang et al. 2011) showed that the apparent viscosity of soy milk increased considerably with an increase in electric field intensity and the number of pulses. The reason might be related to increased interaction among denatured proteins, and the formation of weak transient networks (Xiang et al. 2011). Viscosity affects sensory properties and consumer acceptability of soy milk. Increasing sample viscosity could significantly lower soy milk astringency (Courregelongue et al. 1999).

Pulsed electric energy technologies as non-thermal treatments minimally affected protein quality such as heat-sensitized components during processing compared to conventional thermal treatments. Additionally, Johnson et al. (Johnson et al. 2010) reported that the pulsed electric field was not able to affect the allergenicity of peanut (Ara h 2,6) and apple (Mal d 3) allergens due to no significant changes in the secondary structures and reactivity. However, pulsed electric field treatment reduced the whey protein allergenicity under high intensities (Vanga et al. 2021). Therefore, this treatment has potential in improving the allergenicity of certain food products, but more research needs to explore on its effects on nutritional components.

13.3.2.3 Microwave-Assisted Extraction

Another technology could be applied to enhance protein extraction is microwave processing. This technology is an electromagnetic wave of frequency in the range of 300 MHz–300 GHz, corresponding to 1 m–1 cm wavelength range (Han et al. 2018). Microwave-assisted extraction is increasingly focused nowadays due to higher yield, consuming less energy, non-toxic, and lower cost of operation (Phongthai et al. 2017). Microwaves promote the dipole rotation of the molecules, leading to the disruption of weak hydrogen bonds. This reaction increases the porosity of the biological matrix, resulting in a better infiltration of solvent into the cell to efficiently release intracellular compounds into the solvent system (Kumar et al. 2021a). It should be mentioned that a high amount of thermal energy can be generated during microwave treatment, thus overtreatment can lead to the degradation of heat-sensitive compounds. Under electromagnetic fields, traditional temperature methods, such as thermistors and infrared temperature meters are not available. However, accurate temperature control is acquired by fiber-optical temperature meter which is the most common method for temperature measurement under electromagnetic fields (Jiang et al. 2018).

Generally, microwaving cannot destroy the primary structure of protein because of the lower quantum energy of microwave than that of chemical bond but has a distinct influence on the secondary and tertiary structures caused by carbon-centered free radicals. Since under microwave radiation, polar groups of protein molecules absorb energy and then free radicals can be generated by the kinetic energy. These active radicals interosculate with amino acid residues accompanied by an orderly arrangement of ions, leading to changes in the secondary and tertiary structures (Han et al. 2018). Ochoa-Rivas et al. (Ochoa-Rivas et al. 2017) evaluated the effect of microwaves to assist alkaline extraction of peanut proteins. The changes in protein secondary structures were observed, more specifically the β-sheet and nonordered structures of protein were higher, but the α-helix and aggregated strands were lower

compared to the protein processed by the traditional alkaline extraction method. Significant changes in the secondary structure by microwave treatment were also observed in proteins such as barley protein (Yan et al. 2014), and soymilk proteins (Vanga et al. 2020). Additionally, the effect of microwaves on protein tertiary structure during extraction was reported in some previous research. For example, a slightly increase in surface hydrophobicity was observed for rice bran protein by microwave-assisted process due to the partial unfolding of proteins and exposure of internally buried hydrophobic units (Khan et al. 2011). In another study, microwave-treated sample from pigeon pea protein had a similar surface hydrophobicity compared to the untreated pigeon pea protein (Sun et al. 2020). Smaller particle size and higher disulfide content was also found after microwave treatment (Sun et al. 2020). These results suggest that microwaves could partially denature protein and form protein aggregates via covalent disulfide bonds.

It is noticed that microwave-assisted extraction not only enhances the protein yield and modifies the protein structures but improves the functional properties compared to conventional extraction. For example, Phongthai et al. (Phongthai et al. 2016) applied microwave-assisted extraction for extracting rice bran protein and the protein yield was higher than that of alkaline extraction by about 1.54-fold. Meantime, a higher value of protein solubility at pH 7 was observed after microwave treatment possibly owing to the increased hydrophilicity. This seemed to be explained by the smaller molecular size of protein reduced by microwave treatment through the non-covalent bond rupture of protein molecules, as well as the newly exposed ionizable amino acids and carboxylic group (Phongthai et al. 2016). Because microwave treatment could somewhat dissociate both intramolecular and intermolecular hydrogenated-bond structures. However, a decrease in water solubility of pigeon pea proteins was found after microwave treatment, which was attributed to the protein aggregation through intermolecular interaction of denatured proteins at high temperatures caused by microwave exposure (Sun et al. 2020). Water and oil absorption capacities can also be changed to varying degrees during the microwave extraction process. Microwave extraction of rice bran protein under 600–1000 W for 60–120 s exhibited a higher capacity to absorb water and oil compared to the alkaline extraction (Phongthai et al. 2016). While Khan et al. (Khan et al. 2011) did not report any changes in the water and oil absorption capacities of the protein isolate from microwave-heated rice bran. Ochoa-Rivas et al. (Ochoa-Rivas et al. 2017) also indicated no significant changes in oil absorption but an improvement in water absorption after microwave-assisted extraction (725 W, 8 min). The reason for changes in the water and oil absorption capacities by the microwave process might be the spatial rearrangement of polar and nonpolar side chains in protein structure (Prakash and Ramanatham 1995). More polar side chains are present at primary sites of the protein−water interface, leading to a high-water absorption capacity. While more nonpolar side chains could improve oil absorption capacity by binding the hydrocarbon chains of lipids. Different microwave conditions and protein sources might contribute to different results. Finally, emulsion and foaming properties were changed during microwave-assisted extraction. For example, emulsion activity and stability were improved for microwave-assisted

extracted rice bran protein and peanut protein, possibly owing to the partial unfolding of protein structure and exposing hydrophobic units, facilitating the formation of a resistant film to resist oil drop flocculation, leading to better to form and stabilize the emulsion (Khan et al. 2011) (Ochoa-Rivas et al. 2017). On the other hand, a decrease in foaming activity and stability was observed (Khan et al. 2011) due to the slower diffusion of protein aggregates to air–water interface and the lack of a thick cohesive layer formation around the air bubble to stable the foam. Although a negative effect of microwave on foaming stability was observed in peanut protein, the foaming activity was improved (Ochoa-Rivas et al. 2017). Because microwave-assisted treatment could promote protein adsorption at the air-water interface to reduce the tension between gas and water. Therefore, it is challenging to control the protein modification in structures and functionalities by controlling the processing conditions.

Based on the above review, protein structures and protein configuration can be altered by microwave treatment, which is also related to the change in epitopes. A decrease of 24.7% in the allergenicity of soy protein isolate was observed by microwaving because of the alteration of secondary structure (Li et al. 2016a). In addition, microwave technology is a kind of thermal treatment, which was demonstrated to be effective in decreasing heat stable and heat-labile antinutrients, such as tannin, phytic acid, hydrogen cyanide, total oxalate, and trypsin inhibitor in velvet bean (Kala and Mohan 2012). However, microwave heating might produce nonuniform temperatures and hot spots, which is also detrimental to food quality and nutrition loss. Thus, it needs to more explore to obtain food products with better sensorial and nutritional qualities. For example, equipment and operation should be designed to get the products with improved quality. The combination of movement equipment, like rotation oven and fluidized bed, and microwave heating (Jiang et al. 2018), might be an effective way to ensure the heating uniformity for high-quality products. Moreover, it might be good for some products to heat with reduced power for long duration.

13.3.2.4 Ultrasound-Assisted Extraction

Ultrasound-assisted extraction is another non-thermal extraction technology for plant protein extraction, achieved by the rapid formation and collapse of gas bubbles induced by the ultrasonic waves on the cell surface of the plant proteins. The resulting micro-streaming and shockwaves generate high shear and mechanical force, causing membrane and cell wall disruption, thus can extract the components from plant cells (Kumar et al. 2021a). The application of ultrasound to assist the extraction of plant protein has multiple advantages including more effective mixing, faster energy transfer, selective extraction, and increased production (Pojić et al. 2018). By optimizing various essential factors (i.e., frequency, power, time interval, temperature, pH, ratio, and ultrasound intensity) of ultrasound conditions, ultrasound-assisted extraction has been reported to more efficiently extract plant-based proteins from peanut, black bean, sunflower, soybean and groundnut (Kumar et al. 2021a; Pojić et al. 2018).

As an extraction method that does not require a high temperature, ultrasound-assisted extraction can reduce the degradation or denaturation of thermo-liable proteins, thus better preserving their functional properties. Malik, Sharma, and Saini (Malik et al. 2017) used a high-intensity ultrasound probe and ultrasound bath to extract protein from sunflower meal. Protein solutions (10% w/v) were treated with an ultrasound probe (20 kHz) and ultrasound bath (40 kHz) for various durations, and the structural and functional properties of the extracted proteins were characterized. The SDS-PAGE profile suggested that the extracted proteins had much lower molecular weight compared to the native proteins indicating the change in the primary structure of protein molecules. The increased surface hydrophobicity and sulfhydryl content were ascribed to the partial unfolding of protein molecules after the ultrasound extraction, which gives rise to the exposure of buried hydrophobic groups. The results also revealed that the ultrasound extraction could significantly improve the functionalities of the protein isolates, including solubility, emulsifying and foaming properties and oil binding capacity, while probe sonication-extracted protein exhibited more pronounced functional properties than that of bath sonication-extracted proteins. A study carried out by Jiang et al. (Jiang et al. 2014) reported the effects of ultrasound extraction on the structural and physicochemical properties of black bean protein isolates under low frequency (20 kHz) at various powers and for different durations. The secondary-structure analysis measured by circular dichroism indicated that ultrasonic treated protein samples showed a decrease in the α-helix content and an increase in β-sheets content, while emission-fluorescence spectra also revealed that a changed tertiary structure of black-bean proteins after ultrasonic treatment. This could be explained by the fact that sonication can induce partial unfolding of the ultrasound-treated proteins, causing the exposure of the hydrophobic areas to the surface of protein molecules. As a result, it was not surprising that increased surface hydrophobicity was observed after ultrasound-assisted extraction. It was also noteworthy that ultrasonic power levels significantly influenced the functionality of the extracted proteins. The authors observed that medium ultrasonic power (300 W) was able to increase protein solubility by breaking down unstable aggregates into smaller soluble peptide segments, while the repolymerization of these segments through noncovalent interactions occurred by high-power (450 W) ultrasonic treatments. Another study carried out by Zhang et al. (Zhang et al. 2014) also reported the utilization of ultrasonic-assisted method to extract peanut protein isolate, and its effect on protein structural and emulsifying properties was also evaluated. Significant increases in emulsifying activity index (EAI) and emulsion stability index (ESI) were observed after just 1 min ultrasonic treatment. Based on the results from SDS-PAGE, intrinsic fluorescence emission spectroscopy analysis and circular dichroism spectra, the primary and secondary structure of the ultrasound-treated peanut protein isolate showed no significant change under different conditions. This was controversial as the change of protein primary and secondary structure induced by ultrasound extraction was found by other researchers. The difference was possibly owing to different ultrasound times, power and temperature were used. As a result, the major contribution for the structure change and emulsifying capacity of peanut protein

isolate in Zhang et al.'s work is from the changes in protein tertiary structure rather than secondary structure (Zhang et al. 2014).

Recent research has reported that ultrasound-assisted extraction has the potential to alter the allergenicity of some food resources, such as soybean and shrimp, by physically changing the conformation of allergens and altering their reactivity (Li et al. 2016a). Li et al. reported that ultrasound treatment could significantly reduce 18.9% of the allergenicity when compared to native soybean proteins not only by influencing molecular interactions such as free sulfhydryl and surface hydrophobicity but also by altering the distributions of secondary structures containing helices, strands, turns and structural parameters including average length of helices and strands (Li et al. 2016a). On the other hand, the application of such technology still has some limitations. Prolonged acoustic agitation from cavitation during ultrasound treatment might cause denaturation of soluble protein fractions, thus reducing the yields of proteins. Hence, optimizing the parameters of ultrasound-assisted extraction for different kinds of plant resources is critical for achieving high yields, while preserving the desirable structural, functional, and nutritional properties of proteins. Also, the effect of ultrasound treatment on the sensory properties of plant protein-based food products should be further addressed. For example, Sales and Resurreccion reported a decreased overall acceptance of ultrasound-treated peanuts compared to untreated peanuts (Sales and Resurreccion 2010). This is owing to off-flavors such as bitter and astringent released from increased concentrations of phenolic compounds, through disruption of plant cell walls by ultrasound treatment (Sales and Resurreccion 2010). Besides, combining ultrasound-assisted extraction with other conventional or non-conventional extraction methods should also be considered a promising strategy to improve extraction yields and protein quality. For example, Zhang, Chen, and Zhang developed a complex method to extract intracellular protein from Chlorella pyrenoidosa involving 60% ethanol-soaking, enzyme digestion, ultrasonication, and homogenization extraction (Zhang et al. 2018). Under the optimized conditions, 72.4% of protein was extracted from the microalgae Chlorella pyrenoidosa using the complex method, significantly higher than the protein yield using single processing methods (Zhang et al. 2018).

13.3.3 Enzyme-Assisted extraction Methods

Enzyme-assisted extraction has been considered an environmentally friendly, sustainable, and high-efficiency method to extract high-quality plant proteins. Two types of enzymes are frequently used together or individually to target high protein yield and superior functionality. The first type of enzyme is designed to degrade the rigid cell wall components, such as pectin, cellulose, and hemicellulose, thus opening up possibilities for efficient cellular protein release. Enzymes, including pectinase and carbohydrases, have been successfully employed to damage the integrity of the cell wall of legumes, oilseeds, and cereals seeds to assist protein extraction (Kumar et al. 2021b; Jung et al. 2006; Rommi et al. 2014; Görgüç et al.

2019; Perović et al. 2020). The second type of enzyme aids in liberating protein from the polysaccharide matrix by protein hydrolysis. Plant proteins have complex structure, for example, plant globulins are often hexamers and trimers with much larger molecular weight as compared to their counterpart in milk, causing difficulties in solubilizing into the solvent. Proteases facilitate plant protein extraction by decreasing the protein size and increasing protein solubility (Sari et al. 2015). Depending on the protein sources, these two types of enzymes may be used individually or sequentially to achieve the highest protein extraction efficiency. Sari et al. showed that the addition of protease allowed 50% of protein extracted from rapeseed as compared to 15% without enzyme (Sari et al. 2013). Rommi et al. used carbohydrases to disintegrate rapeseed cell wall and increase the protein extraction by up to 1.7-fold in comparison to non-enzymatic treatment (Rommi et al. 2014). In Niu et al. study, carbohydrases cocktail Viscozyme L and protease Alcalase was used in combination to achieve 82% extraction yield of protein from rapeseeds (Niu et al. 2012). Enzymatic extraction can be used alone or conflated with other extraction methods, such as alkaline extraction, ultrasound, and microwaves (Nadar et al. 2018; Sari et al. 2015).

Cell wall degrading enzymes, such as pectinase, cellulase, xylanase, and glucanase, enhanced protein extraction under mild conditions, which also helped maintain the native protein structure and functionality (Kumar et al. 2021b). The plant protein extracted with this enzymatic method showed high thermal stability since the native protein structure was preserved (Kumar et al. 2021b). Perović et al. used single and multiple carbohydrases, including cellulas, pectinase, and xylanase to help disintegration of the cell wall to facilitate the release of protein into solvent and consequently to enhance soy protein extraction (Perović et al. 2020). Using cell wall degrading carbohydrases can achieve a protein yield comparable to alkaline extraction (pH 8, 2 h, 50 °C) but avoid the drawback of using alkaline. The same protein fractions were extracted from soy grit by alkaline and enzyme-assisted extractions as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), indicating that no protein hydrolysis happened (Perović et al. 2020). The soy protein extracted by enzyme assisted method possessed ameliorated solubility which was attributed to the native structure of soy protein preserved by this mild extraction method. The higher solubility then further contributed to better foaming and emulsifying properties (Perović et al. 2020).

The usage of single or multiple proteases may produce protein hydrolysates with improved functionalities, biological properties (such as metal chelation, antioxidative, antibacterial, antidiabetic, etc.) and nutritive value for various applications. The effects of hydrolysis on protein functionality are on a case-by-case basis, depending on the enzyme, degree of hydrolysis, and the nature of protein. Hydrolyzing protein significantly changes the primary structure of protein, which consequently alter the secondary, tertiary, and quaternary structure of protein. After hydrolysis, protein partially unfolds and exposed its relatively hydrophobic core to the aqueous environment, resulting in changes in its surface hydrophobicity. The surface hydrophobicity further determines influences the interfacial properties of protein, such as emulsifying and foaming properties. A small molecular weight and

balanced surface hydrophobicity may allow protein to diffuse and adsorb to air–water and oil–water interface quicker, which reduces the interfacial tension more rapidly (Lu et al. 2016). Moreover, due to hydrolysis and denaturation, active groups of protein may be exposed and promote protein-protein interactions to form a network, resulting in better gelling properties (Nieto-Nieto et al. 2014). For example, protease M was used to enhance the extraction of soy protein. The hydrolysis resulted in plenty of soluble protein aggregates and small molecular weight hydrolysates. The intrinsic fluorescence profiles showed that during enzymatic extraction, the aromatic amino acid residues in soy protein experienced a gradual transition to a more hydrophilic environment, indicating the partial unfolding of protein (Lu et al. 2016). The enzymatic hydrolysis resulted in more flexible protein with higher surface hydrophobicity, leading to superior emulsifying properties by fast absorption to the oil-water interface and rapid reduction of surface tension (Lu et al. 2016). Moreover, Hamada (Hamada 2000) found that the flavourzyme-assisted extraction resulted in rice bran proteins with enhanced functionality as compared to alcalase-assisted extraction at similar degree of hydrolysis. Flavorzyme-treated rice bran protein hydrolysate showed improved solubility and emulsifying property at pH 5, 7, and 9. The anion exchange separation of the rice bran protein hydrolysates showed that possible deamidation happened during flavourzyme-assisted extraction, which created more negative changes on the surface of the protein hydrolysate and led to increased solubility. Moreover, flavorzyme is a mixture of endo- and exoproteases while alcalase contains endoprotease. Flavorzyme is considered to have the ability to cleave off the hydrophobic amino acid residues at the end of the polypeptides, which contributes to the improved solubility. This study shed interesting light on the fact that not only the degree of hydrolysis but also the enzyme cleaving positions plays important role in protein functionality (Hamada 2000).

The protease-assisted extraction would be a credible method to concurrently improve plant protein yield and modify their functionality for a wide spectrum of applications (Kumar et al. 2021b). For example, the high solubility and enhanced functional properties of plant protein hydrolysates allow them to be used in many foods as functional ingredients, such as beverages, pourable and unpourable dressings, coffee creamers, whipping creams, bakery, soups, and meat analogs or substitutes. Moreover, it has been reported that some plant protein hydrolysates with controlled hydrolysis degree have health-promoting benefits, such as antioxidative, anticancer, and antidiabetic, which allow them to be value-added ingredients in a wide variety of functional foods (Mccarthy et al. 2013). Furthermore, plant protein extracted with partial hydrolysis may have improved sensory properties. Due to the mild extraction condition, the appearance of the protein may be lighter and brighter than the counterpart isolated by alkaline-isoelectric precipitation, resulting in an increasing acceptability to consumers. Additionally, some plant polypeptides have flavor and taste enhancement effects, which makes them more attractive to consumers (Wang et al. 2020). However, the protein hydrolysis reaction must be controlled since the hydrolysis degree proceeds beyond the optimal stage will result in bitter taste and unfavorable functionalities. There is a balancing point between

extraction efficiency and optimal functionality. More research is needed on this topic since the optimal condition depends on the plant sources, the type of enzyme, and the targeted functionality and applications.

Currently, there are still some challenges that prevent the wide application of enzyme-assisted protein extraction methods. Examples include, but are not limited to, high solvent consumption, longer reaction time, and high enzyme price. Some strategies have been proposed by academic research to further increase the efficiency of enzyme assisted protein extraction method and overcome some of its drawbacks. Firstly, the invention of immobilized enzymes may not only significantly reduce the cost of enzyme-assisted protein extraction, but also contribute to a more simple and sustainable process (Nadar et al. 2018). Secondly, enzyme-assisted extraction can be strategically integrated with other non-conventional extraction techniques, such as microwave, ultrasound, or high pressure to treat complex plant matrices. Each extraction technique has its own advantages and disadvantages. For example, ultrasound-assisted extraction is fast, relatively low cost, and has the potential to scale up, but prolonged and excessive sonication treatment may reduce the protein yield and lower the protein quality by stimulating the formation of insoluble protein aggregations (Kumar et al. 2021b). High pressure-assisted extraction techniques can cause cell deformation and facilitate solvent penetration and protein release at ambient temperature, as well as inactivate microbes and enzymes without affecting the sensory property of the protein. Other benefits of using high pressure include but not limited to shortening extraction time, reducing solvent consumption, and increasing protein purity. However, depending on the duration and strength of the high pressure, the protein structure and functionality may be altered (Xi, J. 2017). Furthermore, pulsed electric field-assisted extraction method is another promising non-thermal technique that can be used to improve protein recovery. However, pulsed electric field technique itself is not comparatively sufficient to achieve high protein yield (Kumar et al. 2021b). Therefore, tactically combining two or more extraction methods may substantially improve the extraction yield and increase the protein quality as well as overcome the drawbacks of each extraction method when used alone.

13.4 Applications of Plant Proteins in Food

Due to the rising awareness about the health-promoting effects and sustainability benefits of plant proteins, more and more plant-based food products have been developed as affordable substitutes and alternatives for those made from animal sources. At this stage, both academia and industry are exploring appropriate methods to extract plant proteins from their original matrices to meet the increasing demands for the ever-growing plant-based food market. The extraction methods exert significant influence on the proteins' yield, purity, functionality, sensory quality, and consequently the applications. The applications of plant proteins can range from nutrition fortifiers, functional ingredients in traditional products, to novel food formulations (such as gluten-free, egg-free, dairy-free, etc.). These cover a wide

variety of food products, from liquid (such as smoothies, juices, plant-based milk alternatives, sport drinks coffee creamers, cuisine creams, infant formulas, etc.), semi-solid (such as yogurt, slushes, ice creams, dips, dressings, soups, and puddings, etc.) to solid (pastas, noodles, spaghetti, bakery, meat analogs, energy bars, etc.).

Depending on the requirement for the specific application, plant protein extract from dry separation or plant protein isolates or concentrates from wet separation methods could be selected. For example, protein enriched fractions had been successfully separated by air classification from sources like peas, lentil, and beans (navy bean, black bean, mung bean, pinto bean) to protein contents of 25 to 60% (Fernando 2021). Pulse protein-enriched fractions by air-classification can be added to cereal food products to increase protein content and improve nutritive value since pulse proteins have relatively higher lysine content than those of cereals. Products like Catelli Protein pasta and spaghetti, made with wheat flour and faba bean protein fraction, have been successfully launched in the market. Dry separated high protein fractions can also be added to food formula as functional ingredients. For example, Gómez et al. attempted to use air-classified pea protein-enriched fractions in layer and sponge cakes to partially replace wheat flour (Gómez et al. 2012). Han et al. used pea protein fractions to prepared gluten-free cracker snacks (Han et al. 2010). These gluten-free crackers showed light color, good flavor and crisp texture, resulting in a high consumer acceptance. As products, such as crackers, cookies and doughnuts, require less ability to retain gas in the structure, protein-enriched fractions could be included in the formulation with less challenges. For products, like breads, the addition of protein-enriched fractions to high proportion may reduce the quality of the dough since the impurities in the protein-enriched fraction may disrupt the cohesive protein/starch network, which reduces the ability of the dough to retain gas.

Plant protein isolates extracted by wet processing are versatile functional ingredients due to their relatively high purity and good functionality. For example, Jarpa-Parra et al. explored the potential of using lentil protein isolate in angel food cake and muffin as egg/milk replacer (Jarpa-Parra et al. 2017). This study suggested that due to the excellent foaming and emulsifying properties of lentil protein isolate, it can totally or partially substitute egg/milk in cake with satisfactory quality (Jarpa-Parra et al. 2017). Additionally, pea protein isolate had been utilized to develop dairy-free dessert like puddings based on their gelling and thickening properties (Nunes et al. 2006). Plant protein isolates can also be used as gelling agents and emulsifiers in meat products, including restructured, coarse ground, and comminuted meats (Drakos et al. 2007; Paglarini et al. 2019). It must be emphasized that plant protein isolates and concentrates from various sources, such as soy, pea, chickpea, and oat, have been utilized in the preparation of meat analogs. The market and plant protein-based meat analog products are thriving (Kumar et al. 2017; Sha and Xiong 2020). Plant protein isolates, concentrates, and/or their texturized products have been formulated into food products that resemble the sensory characteristics of certain types of meat.

In response to the growing trend of meat analogs, animal milk alternatives and other plant-based food producs, the food industry is looking for high-quality plant proteins. Though novel technologies have been developed and under continuing

research, most of the plant protein isolates used in industry are currently prepared by conventional methods mainly alkaline-isoelectric precipitation. These conventional solvent extraction methods are simple and provide relatively high yield. However, the financial and environmental costs of wet processing are comparatively higher than dry separation, which limits the utilization of plant protein isolates and concentrates to relatively high-price products and/or elevates the plant-based food product price (Zhu et al. 2021). One of the major challenges of plant protein isolates/concentrates prepared by conventional methods are their organoleptic properties (Zha et al. 2019). Depending on the plant sources, conventional isolation methods, especially alkaline treatment, may lead to protein products with undesirable flavor, taste, and color. It has been demonstrated that using novel technologies (such as high pressure, microwave, ultrasound, pulse electric field and enzyme-assisting, etc.) along or in combination with conventional methods has potential to achieve high protein yield, improved sensory quality, and functionality, which will increase the palatability and consumer appeal of plant protein isolates/concentrates (Görgüç et al. 2019; Perović et al. 2020; Zhao et al. 2022). For example, combining enzyme-assisted extraction technique with traditional extraction methods may extract a high yield of protein at mild conditions while maintaining protein's native structure, contributing to better protein solubility, which allows the protein product to be potentially used in beverage products.

13.5 Conclusion

The present chapter summarized the effects of conventional and non-conventional extraction techniques on protein structures and functional properties. Novel emerging extraction technologies were highlighted as they can be seen as an alternative for conventional technologies with the aim to improve extraction efficiency and the quality of the plant protein extracts with less environmental burden. In some cases, these innovative extraction methods enhanced the functional properties of extracted proteins. For instance, novel solvent extraction methods like RME can remain the native conformation of the proteins and offer protein extracts with improved functional properties (e.g., gelling, foaming, and emulsifying capacities) as well as nutritional properties. Energy-assisted extraction methods, including high pressure, pulsed electric field and high voltage electrical discharge, microwave, and ultrasound, can increase protein yields and enhance the solubility, water and oil holding capacities, emulsifying, foaming, and other functional characteristics of the extracted plant proteins under their optimized processing parameters. Although these innovative technologies showed merits over conventional protein extraction methods, the majority of the reviewed examples in this work and the obtained results were carried out at a laboratory scale. In the last years, some novel extraction technologies, such as pulsed electric field- and high pressure-assisted extraction, have been transferred to large-scale industrial applications, while the majority of these technologies are still in their infant stage for their commercial applications, owing to the reason that based on lab experiments it is

hard to estimate if the industrial process will be cost-effective and economically viable. More research is needed to promote the further utilization of these novel technologies. For example, studies are devoted to overcome the challenges of enzyme-assisted protein extraction, such as long processing time, high operational costs, high energy consumption, and the necessity of a careful adjustment of pH and temperature by combining with other non-conventional technologies, such as microwave and ultrasound. Recent studies also demonstrate that using novel technologies (such as high pressure, microwave, enzyme-assisting, etc.) along or combining with conventional methods has the potential to achieve improved sensory quality, which will increase the palatability and consumer appeal of plant protein isolates. Future work should focus on developing cost-effective and sustainable extraction methods as well as improving the consumer appeal of plant protein-based products with the aim to enlarge the supply and utilization of plant proteins.

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