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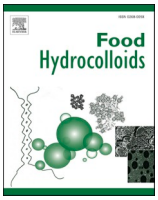

Plant protein aggregates induced by extraction and fractionation processes: Impact on techno-functional properties

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ABSTRACT

Currently, plant proteins are fractionated to ingredients with high purities, but an often ignored point is the impact of the extraction and fractionation process on protein functionality. To allow a fair and effective comparison, it is key to understand the changes in protein's aggregated state occurring in the extracted ingredients during processing. We review conventional and upcoming plant protein extraction and fractionation processes (on pulses and oilseeds) and focus on how the processing history influences the macroscopic functional properties of the proteins. To establish this link, we dive into seed morphology and give an overview of the plant seed composition. In addition, we explain the essence of each process step and how it impacts the protein's aggregated state. The latter is linked to the macroscopic functionality (foaming, emulsification, and gelation). We identified three major protein structure-changing steps in the conventional protein extraction process: defatting, alkaline extraction, and isoelectric point precipitation. These steps lead to large, insoluble aggregated structures, which strongly impacts the protein macroscopic functionality. Milder extraction methods reduce these alterations, but a potential consequence is the presence of non-proteinaceous components, which could give challenges in sensory and nutritional aspects and affect the techno-functional properties of the ingredient. The take-home-message is that we need to consider the process-induced change of the protein aggregated structures, which are likely to dominate the functionality over the protein's molecular parameters.

1. Introduction

With a growing global population, food demand is likely to increase while at the same time, raw materials, energy and water become scarcer (Aiking, 2011). As a result, we require more sustainable ingredient sources and production methods for our foods. A solution may lie in shifting the balance to the consumption of plant-derived proteins instead of animal-derived proteins, as animal-derived foods generally involve more resources and generate higher environmental stress (Aiking & de Boer, 2018; Friel et al., 2009).

Proteins are an inherent part of our diet. Next to their nutritional role of providing amino acids, they also possess many techno-functional roles. Proteins are crucial in forming or stabilising relevant structures in foods, such as aggregates, droplets, bubbles and gels. In order to produce plant protein ingredients, extraction from plant crops (mainly on pulses and oilseeds), followed by fractionation, is generally required to obtain ingredients with high protein purity. In this review, the terms extraction and fractionation will be used to describe the processes, where extraction refers to the process where components are extracted from the seed matrix, and fractionation refers to the separation process,

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where components are separated into different fractions. These processes are necessary, as plant cells in seeds are relatively dry and plant proteins are compartmentalized to prevent interactions (Okita & Rogers, 1996). During an aqueous extraction processes, the proteins are exposed to water and multiple types of interactions (e.g. hydrophobic and electrostatic forces). Fractionation is then necessary to obtain high purity protein ingredients, which uses conventional fractionation processes to remove non-proteinaceous components, which require organic solvents, pH shifts and heat (to evaporate residual solvents, water and ensure microbial safety) (Sari, Mulder, Sanders, & Bruins, 2015). These additional processing conditions might further enhance protein interactions and often result in substantial chemical (e.g. phenol-protein interaction) and structural (e.g. denaturation) changes of the proteins. A consequence of these changes is the formation of larger aggregated structures, such as insoluble protein aggregates, which may affect functional properties at the colloidal (or meso-scopic) scale (Kornet, Veenemans, et al., 2021).

The techno-functional properties of plant proteins have been widely studied, and several reviews were published, focussing on gelation and multiphase systems (Amagliani, Silva, Saffon, & Dombrowski, 2021; Drusch, Klost, & Kieserling, 2021; Hinderink, Boire, et al., 2021; Yong, Sim, Srv, & Chiang, 2021). However, the impact of extraction processing on plant protein functionality is not always considered. As a result, protein extracts obtained from the same source but with different extraction processes are compared, while the comparison might not be straightforward due to process-induced alterations, such as differences in protein composition (e.g. albumin and globulin ratio) and aggregated structures. These changes may largely affect macroscopic techno-functional properties, such as foaming, emulsifying and gelling.

The link between extraction processing and techno-functionality is not always evident, as there is no consensus on how and which molecular or colloidal properties link to techno-functionality. On the molecular side, there is a biochemistry- and bioinformatic-based approach, by annotating the protein amino acid sequence and 3D structure (Nietzel et al., 2013; Perera, McIntosh, & Wanasundara, 2016). This structure is then linked to protein properties, such as charge and hydrophobicity; that affect in turn the protein colloidal properties. This amino acid to protein structure-function approach aims to link functionality to protein molecular properties of the specific proteins. This view on protein functionality would be valid for highly pure single native protein systems, often obtained using extensive purification steps using chromatography (Bérot, Compoint, Larré, Malabat, & Guéguen, 2005). On the other end, there is a physics perspective that might oversimplify the protein structure (e.g. into spheres) to be able to establish multi-length scale relationships (Poirier et al., 2022; M. Y. Shen, Davis, & Sali, 2005). Another upcoming idea is the increasing role of aggregated structures (e.g. quaternary structures or sub-micron-aggregates), which might play a dominating role in the techno-functional properties, over molecular properties. Here, the composition of proteins could also be important, as the various proteins present in seeds might aggregate differently upon processing, which in turn affect functionality.

This brings us to the aim of this review, which is to highlight how extraction processes affects plant protein techno-functionality. We will do this by building up the process-functionality relationship in a stepwise manner, as one should understand the combination of plant seed morphology, extraction/fractionation processing and the mechanistic understanding of functionality in order to comprehend the impact of processing on protein functionality. First, we describe the plant seed morphology, which is key to understanding each process step. We dissect the conventional protein fractionation method into individual steps, where we discuss the essence of each step and how it impacts the composition and aggregated structures of the final protein ingredients. Finally, we will discuss how key protein-altering steps can be changed to improve protein techno-functionality. Such an alternative approach could be mild fractionation, which involves fewer processing steps, lower temperatures, and minimalise organic solvents than conventional fractionation. A milder treatment may reduce the impact on the protein structure (Kornet et al., 2020; Lie-Piang, Yang, Schutyser, Nikiforidis, & Boom, 2023), thus macroscopic techno-functionality, which will be elaborately addressed.

2. Plant seed morphology and protein composition

2.1. Seed composition and morphology

Proteins can be extracted from different type of sources, and in this work, we will focus on two major plant protein sources which are commonly used for extraction of (partly) water soluble proteins: pulses and oilseeds. For fair comparisons between the cited references, we recalculated all the mentioned protein contents in this work using a nitrogen conversion factor of 5.7 (Keuleyan et al., 2023). Pulses are part of the leguminous family; examples are pea, lentil and mung bean. They are generally high in carbohydrates (mainly starch, 20–59 wt%) and proteins (14–31 wt%), and have a low-oil content (1–7 wt%) (Hall, Hillen, & Robinson, 2017). Not all members of the leguminous family are high in starch, and examples are lupin (~40 wt% protein, 7.5–9.3 wt % oil, 40 wt% fibres, 0.2 wt% starch) (Al-Amrousi et al., 2022; Bryant, Rangan, & Grafenauer, 2022; Lo, Kasapis, & Farahnaky, 2021) and soybean (35–40 wt% protein, 20 wt% oil, 9 wt% fibres, 11–12 wt% starch) (Qin, Wang, & Luo, 2022; Stevenson, Doorenbos, Jane, & Inglett, 2006). Structure-wise, soybeans have more similarities to oilseeds, and examples of oilseeds are sunflower, flaxseed and rapeseed, which have high oil contents (45–65 wt%) but also contain proteins (20–35 wt%) (Gonzalez-Perez & Vereijken, 2007; Wanasundara, 2011).

Understanding the structural organisation of proteins and other components (e.g. lipids and starch) in plant cells is crucial to designing protein extraction and fractionation processes (Tamayo Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). For this, several factors have to be considered, such as the cell architecture, the interconnection between cell components, and the biochemical differences between plant sources (e.g. type of proteins and non-proteinaceous components).

In plant seeds, storage proteins are contained in organelles called protein bodies (PB), which are present in pulses and oilseeds as small spherical shapes (Fig. 1) (Hoglund, Rodin, Larsson, & Rask, 1992; Murphy & Cummins, 1989) and protect the proteins until further use upon seed germination. Protein bodies comprise 70–80% of the storage proteins and have a diameter of 1–20 μm enclosed by, in most cases, a single phospholipid membrane on the outside (Pelgrom, Boom, & Schutyser, 2015; Pernollet, 1978). The storage proteins are mainly stored in crystalline form in the so-called crystalloids with a diameter of approximately 15 nm, of which proteins need to be extracted from, typically using aqueous extraction. In addition, the protein bodies contain the antioxidant phytic acid (10 wt%), (inactive proteolytic) enzymes, cations and ribonucleic acids (Pernollet, 1978). Especially the phytic acid may play a large role in protein aggregation, which will be addressed in section 3.3.

In addition to protein bodies, most pea seeds contain starch granules (SG, black oval shapes in Fig. 1A). A starch granule is a semi-crystalline storage organelle for starch molecules with a diameter between ~5 and 62 μm (Singh, 2021). In contrast to peas, rapeseeds contain low amounts of starch, but high amounts of lipids that are stored in so-called oleosomes (OL), also known as oil bodies or lipid droplets (small dark spheres in the image of the rapeseed (Fig. 1B)) (Thiam, Farese, & Walther, 2013). Oleosomes are oil storage organelles, where lipids are enclosed in a monolayer of phospholipids and structural proteins, forming spherical emulsified oil droplets in an aqueous phase (Abdullah, Weiss, & Zhang, 2020; Plankensteiner et al., 2023). The enclosure of lipids into these structures has been developed by nature to store energy and provide lipids (triacylglycerols) with high physical and chemical stability (Nikiforidis, 2019).

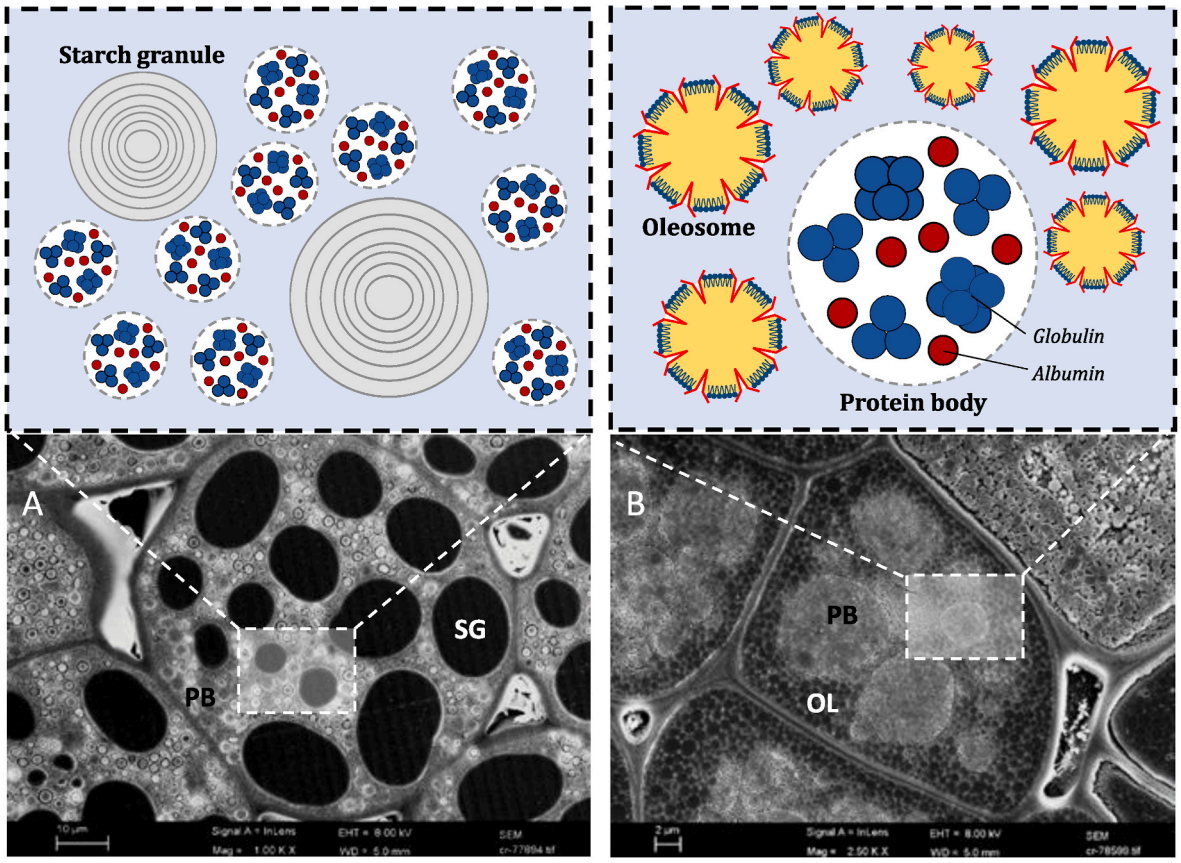

Fig. 1. The plant cell of pea (A) with starch granules (SG) and protein bodies (PB), and of rapeseed (B), with protein bodies (PB) and lipids (oleosomes) (OL), visualised using electron microscopy. The top-view panels represent a simplified scheme of the main native organelles present (not to scale). Panel A is unpublished data from our lab. Panel B is obtained from (Kornet et al., 2020). The scale bars correspond to 10 μm (A) and to 2 μm (B).

2.2. Plant protein composition and structures in seeds

It is crucial to understand the protein's present in the seeds, as their intrinsic properties are exploited during extraction. This section introduces the type of proteins present (see for more extensive reviews (Damodaran, 1997; Foegeding & Davis, 2011; Kinsella, 1976; Moure, Sineiro, Domínguez, & Parajó, 2006)) and provides an overview of the protein composition in pulses and oilseeds.

The storage proteins are divided into four protein classes, which are based on their solubility using the Osborne classification: (1) water-soluble albumins, (2) dilute saline-soluble globulins, (3) ethanol-soluble prolamins and (4) dilute acid/alkaline soluble glutelins. This classification indicates the optimum condition for solubility but is not conclusive, as the proteins are amphiphilic and charged molecules and their solubility can depend on different factors, such as ionic strength and pH. The water-soluble albumins and dilute-saline-soluble globulins are the most abundant in pulses and oilseeds.

Plant proteins are often classified using another method, which is by their size, using Svedberg (S) units. This coefficient indicates the sedimentation speed of particles under acceleration, in this case proteins, where a lower S-unit shows slower sedimentation, due to a smaller protein size, when assuming a constant protein and solvent density. Examples are 2S for monomers, 7S for trimers, and 11 or 12S for hexamers. This classification is quite often assumed for each protein, and might not always be true as environmental conditions (e.g. pH and ionic strength) determine the quaternary structures and also the formation of larger aggregates (Gonzalez-Perez & Vereijken, 2007). Therefore, one should be careful when using the S units for plant proteins.

The protein sizes and structure of both albumin and globulins are pronouncedly different. Generally, one or two major albumin groups are present in pulses and oilseeds with Mw between 18 and 24 kDa (Souza, 2020). For instance, the two main pea albumins are known as pea albumin 1 (PA1) and 2 (PA2) with Mw of 6 and 26 kDa, respectively (Lu, Quillien, & Popineau, 2000). Two well-characterised oilseed albumins are the ones of sunflower seed and rapeseed, known as sunflower albumin (SFA, 10–18 kDa) and napin (17 kDa) (2S), respectively (Gonzalez-Perez & Vereijken, 2007; Rico, Bruix, González, Monsalve, & Rodríguez, 1996).

The globulins show a different behaviour. For example, the monomer of pea globulin (legumin, 11S) has a Mw of approx. 57–62 (O'Kane, 2004). Around pH 7.0 and low ionic strength, these monomers are associated into a hexameric structure (Fig. 3) (±300 kDa) (Shewry & Casey, 1999; Tandang-Silvas et al., 2010). Under similar conditions, another type of pea globulin called vicilin has a trimeric structure (7S). A vicilin monomer has a Mw of approximately 47–50 kDa (±150 kDa). The quaternary structure of vicilin and legumin are pH- and ionic strength-dependent due to a combination of electrostatic and hydrophobic forces. For instance, at acidic pH (<3), native globulins of pea, rapeseed and sunflower seeds exist in monomeric form (2S) (Gonzalez-Perez & Vereijken, 2007; Perera et al., 2016). Globulins generally possess an isoelectric point (pI) between pH 4.0 and 5.0 (Chéreau et al., 2016; Sari et al., 2015). As a result, they have low solubility around the pI and higher solubility at pH values below 3.0 and above 7.0.

An overview of the albumin and globulin composition of several plant seeds was previously given by Chereau et al. (Chéreau et al., 2016) and Mouzo et al. (Mouzo, Bernal, López-Pedrouso, Franco, & Zapata, 2018), which we summarised in Table 1. In this case, the prolamin and glutenin fraction of the seeds was not included. In addition, we added the corresponding Svedberg units and their specific nomenclature, if applicable.

Table 1
Overview of plant seed protein composition (wt%) based on solubility (Osborne's) classification and sedimentation coefficient. The albumin and globulin composition is only reported, while the prolamin and glutelins are not shown. Values retrieved from (Chéreau et al., 2016; Mouzo et al., 2018).

	Albumin content (wt%)	Globulin content (wt%)
Soybean	10%, 2S	65–90%, β-conglycin (7S), glycinin (11S)
Pea	20%, 2S	65%, vicilin (7S), legumin (11S)
Faba bean	12–25%, 2S	50–55%, vicilin (7S), legumin (11S)
Sunflower	20–62%, 2S	38–60%, heliathenin (11S)
Rapeseed	50–60%, napin (2S)	20–25%, cruciferin (11S)

For the mentioned pulses and oilseeds, >60% of the total seed protein composition comprises of albumin and globulin proteins (Table 1). The ratio largely depends on the cultivar type and environmental growing conditions (Spencer & Boutler, 1984). An example is the cruciferin-to-napin (globulin-to-albumin) ratio in rapeseeds, which may range from 1.4 to 0.1 (Stolte, Vettel, & Möllers, 2022). While oilseeds seem to mainly contain one major family of globulins, such as cruciferins for rapeseed and heliathenins for sunflower seeds (Gonzalez-Perez & Vereijken, 2007), pulses can have multiple types of globulin proteins, such as legumins and (con)vicilins. The legumin-to-vicilin ratio in peas can vary between 2.8 and 0.1, depending on the cultivar (Barac et al., 2010; Casey & Domoney, 1999; Gueguen, Chevalier, And, & Schaeffer, 1988; Vreeke, Meijers, Vincken, & Wierenga, 2023).

3. Plant protein extraction and fractionation

The mainstream protein extraction and fractionation process for pulses and oilseeds is a conventional process, of which a schematic overview is shown in Fig. 2. This wet process is also known as the (conventional) alkaline extraction-acid precipitation method. We will discuss each processing step in detail and show their impact the protein functional properties.

3.1. Processing of seeds before protein extraction

Pre-processing of the seeds facilitates the wet extraction steps later in the process by increasing the water extractability of the proteins. A general first step is dehulling, as the hulls are high in non-proteinaceous components, such as fibres and phenols (N. Wang, 2008). The second step is milling to remove the physical barrier (protein bodies entrapped in partly cell wall structures) and to obtain particle sizes smaller than that of the intact cells, thereby facilitating the extraction of the water soluble components, including proteins, during the alkaline wet extraction step (Preece, Hooshyar, & Zuidam, 2017). We refer to previously published work for more information on this topic (Preece et al., 2017; Rosenthal, Pyle, & Niranjan, 1998; Russin, Arcand, & Boye, 2007; Vishwanathan, Singh, & Subramanian, 2011).

For oilseeds, a defatting step is generally performed before milling, to maximise lipid extraction yields and obtain lipid free protein fractions. Oil is also removed as it may reduce the efficiency of the milling step. The industrial oil extraction process contains flaking, oven/steam heating, pressing, solvent extraction (often hexane) and desolventisation steps (McCurdy, 1990; Mosenthin et al., 2016; Nehmeh et al., 2022). The result is an effective extraction, with about 0.2–2 wt% of residual oil left in the defatted meal (Fetzer, Herfellner, Stäbler, Menner, & Eisner, 2018; Preece et al., 2017). This meal (also known as defatted cake) can be rich in proteins with e.g. 35–40 wt% based on dry matter for rapeseed meal and 44–54 wt% for soy bean.

Extensive heating steps are necessary to inactivity enzymes, kill microbes, destabilise oil droplets, remove volatile anti-nutritional compounds (e.g. glucosinolates) and evaporate solvents. A drawback is the denaturation of proteins, followed by protein-protein crosslinking, aggregation of proteins, thus reduced protein solubility (McCurdy, 1990). As a result, low protein extraction yields are achieved, and resulting fractions have a dark-colour with lower protein solubility (Fetzer et al., 2018, 2019). A final drawback of extensive heating is the induction of protein oxidation; a covalent modification of proteins that are generally induced by reactions with reactive oxygen species. Such a reaction can lead to lower protein digestibility, but also protein aggregation or even breakdown of protein structure (Poojary & Lund, 2021). One should keep these potential protein alterations in mind when using industrially defatted cakes from oil-rich seeds.

A sidenote here is that defatting is typically performed on high oil content seeds. Low oil content seeds, often pulses, are generally not defatted. As a result, lipids are co-extracted with the proteins and are present in the ingredients. For example, lipids comprise up to 10% and 3% for pea and lupin protein isolate, respectively, and 5 and 10% for pea and lupin protein concentrates, respectively (Keuleyan et al., 2023).

3.2. Alkaline wet extraction

The second phase is the protein extraction step, where the (defatted) flour is dispersed in high volumes of water, sometimes at elevated temperatures up to 60 °C (Lam, Can Karaca, Tyler, & Nickerson, 2018). Flour-to-water mass ratios ranging from 1:6 to 1:10 have been used (Möller, Li, van der Goot, & van der Padt, 2021). An alkaline extraction pH would further protonate proteins, giving them a negative charge. This hydration and newly introduced hydration forces would allow the diffusion of proteins from the protein body to the solvent (Kornet, Penris, et al., 2021). The aqueous solvent is generally suitable to extract the water soluble albumin proteins, while only parts of the globulin proteins are soluble in water-only systems. Therefore, the extraction pH

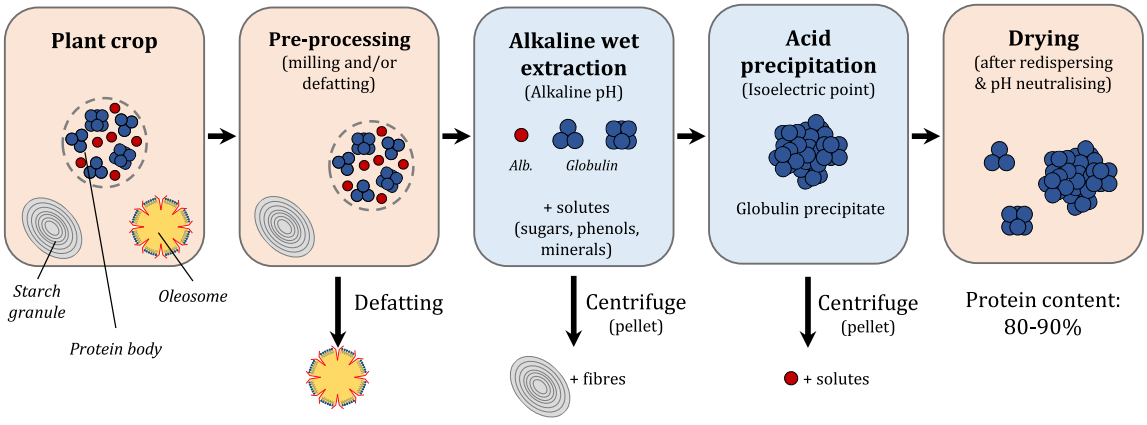

Fig. 2. Schematic overview of the conventional wet protein fractionation methods.

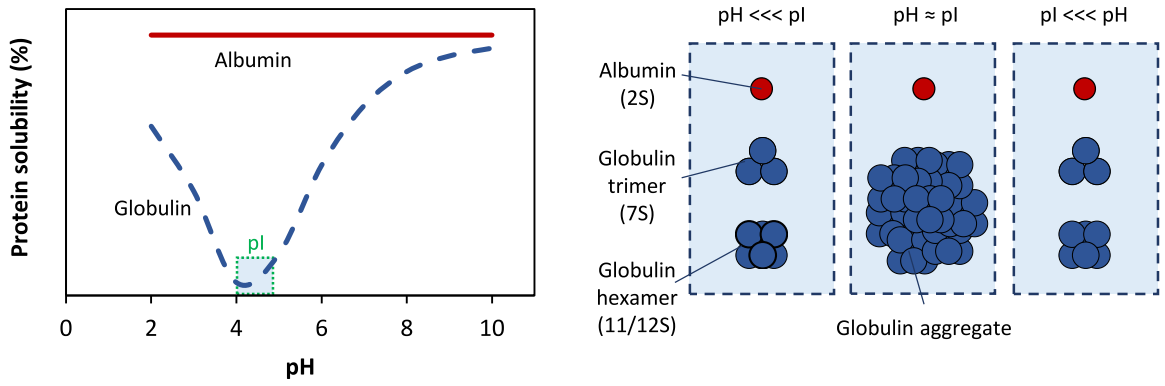

Fig. 3. *Schematic overview of typical solubility curves of albumin and globulin on* (Gonzalez-Perez, Vereijken, van Koningsveld, Gruppen, & Voragen, 2005). *Albumin is shown as the red solid line, while globulin is shown as a blue dashed line (left), and their quaternary structure as a function of* pH *(right).*

is often alkaline, with pH values up to 13.0, which is far away from the globulin's iso-electric point, leading to high protein surface charges, thus an increase in globulin protein solubility (Fig. 3).

Although alkaline extraction conditions improve the protein extraction yield, there is a potential risk of further implications on protein structure and functionality (Manamperi, Wiesenborn, Chang, & Pryor, 2011; McCurdy, 1990). This is intimately related to the solubilisation of non-protein components, such as sugars, minerals and phenolic compounds. Especially the phenolic compounds have a significant impact on the proteins at alkaline pH by (non-)covalent interactions (Keppler, Schwarz, & van der Goot, 2020). There is considerable variation between pulses, with lentils, red kidneys and black beans having a 3–4 times higher phenol content than green peas, yellow peas and chickpeas (Campos-Vega, Loarca-Piña, & Oomah, 2010). Also, within sources, a variety of protein composition and phenol content is present due to genetic selection and the growing conditions. Oilseeds are often rich in phenols with contents up to 2 and 3% of the total seed content for sunflower seed and rapeseed, respectively (Karefyllakis, Salakou, Bitter, van der Goot, & Nikiforidis, 2018; Wanasundara, 2011).

Phenols are generally highly soluble (and extractable) at neutral pH of 7, and solubility might slightly increase further when increasing pH to 8 (Blum, 2019; Sripad, Prakash, & Rao, 1982). The phenols can be oxidised into reactive products, known as quinones (Cai, Arntfield, & Charlton, 1999; Shahidi & Senadheera, 2019), which bind irreversibly and covalently to proteins (i.e. complexation). Autoxidation of phenols can occur at neutral pH, but the oxidation rate is higher at more basic pH values. The rate of oxidation and pH threshold where the reaction is initiated differs for each phenol type (Keppler et al., 2020).

A result of protein-phenol oxidations is the alteration of the protein secondary and tertiary structure (Rawel, Meidtner, & Kroll, 2005), as demonstrated for sunflower seed, where proteins covalently bind to the main phenol, chlorogenic acid (Karefyllakis et al., 2018). Protein-phenol interactions may lead to protein-phenol complexation, thus forming large aggregated structures and reducing solubility (Pringent, Voragen, Visser, van Koningsveld, & Gruppen, 2007; Wei, Yang, Fan, Yuan, & Gao, 2015). Phenol oxidation may lead to dark colour formation of the protein fractions (McCurdy, 1990).

The final step of the alkaline extraction is the separation of the soluble components from the insoluble fraction, containing starch granules (for pulses) and cell wall material. The dispersion is centrifuged or filtrated on a lab scale, such methods are energy-intensive and sometimes impractical at large scales. An additional method for industrial-scale is, for instance, decanting (Weisz, Schneider, Schweiggert, Kammerer, & Carle, 2010). The result is a supernatant, rich in soluble (non-)proteinaceous components.

3.3. Acid precipitation of proteins

The proteins in the previously mentioned supernatant are generally fractionated using an acid protein precipitation step to separate proteins from the soluble non-proteinaceous components (e.g. sugars, minerals and phenols) using centrifugation, filtration or decanting (Manamperi et al., 2011; Tzeng, Diosady, & Rubin, 1990). The pH of the supernatant is reduced close to the pI of the plant globulins (often pH = 4–5), where the proteins have a net-zero charge (Fig. 3). If weak to no repulse electrostatic forces are present, the non-polar amino-acid groups will aggregate leading to protein aggregation, and finally precipitation of plant globulins (Chéreau et al., 2016). T. The aggregation is strong for globulins, due to high surface hydrophobicity, especially compared to albumins. For pea and Bambara groundnut proteins, globulins were shown to have three to nine times higher surface hydrophobicity than albumins (Kornet, Veenemans, et al., 2021; J. Yang, de Wit, et al., 2022). The reason could be the presence of more hydrophobic domains on the protein backbone for globulins than albumins, as shown for rapeseed proteins (Ntone et al., 2021; Perera et al., 2016; Rico et al., 1996).

After acid precipitation and a gravitational/filtration step, the protein-based pellet can be further purified by multiple washing steps. The pellet is often redispersed, pH neutralised, and subsequently (freeze or spray) dried, yielding a protein fraction of 80–90% protein purity (Chmielewska et al., 2020; Sari et al., 2015). The protein yield of this globulin-dominated ingredient will vary depending on several earlier mentioned process steps, such as protein denaturation upon defatting, cell wall disruption upon milling and extraction pH. As a result, the protein yield values can have a wide range from 16 to 80%, for sunflower seeds, pea, Bambara groundnut, mung bean and soy (L'Hocine, Boye, & Arcand, 2006; Pickardt, Eisner, Kammerer, & Carle, 2015; J. Yang, Kornet, et al., 2022).

Additionally, the acid precipitation step has several disadvantages. The first one is the large aqueous side-stream with soluble components, including albumin proteins (Chua & Liu, 2019). This albumin-rich fraction is usually discarded, resulting in a loss of a valuable ingredient, as the albumin proteins can comprise up to 30 wt% for pulses (Hall et al., 2017) and 40 wt% for oilseeds (Gonzalez-Perez & Vereijken, 2007; Souza, 2020; Wanasundara, 2011). Typical protein yields for lupin, rapeseed, pea, Bambara groundnut and mung bean albumins were in the range of 10.5–18% (Bérot et al., 2005; Wong, Pitts, Jayasena, & Johnson, 2013; J. Yang et al., 2023; J. Yang, Kornet, et al., 2022). Another disadvantage of acid precipitation could be the potential risk of irreversible globulin aggregation at the proteins pI, which is shown for yellow pea (Kornet et al., 2020), Bambara groundnut (J. Yang, de Wit, et al., 2022) and faba bean protein isolates (Langton et al., 2020). If the aggregates are large enough, this could lead to lower protein solubility,

which is shown for yellow pea protein, where the protein solubility reduced from 94 to 79% after isoelectric point precipitation (Kornet, Shek, et al., 2021). For soy, a similar result was shown, where the protein solubility reduced from 45-68% to 15–37% after precipitation (Rao, Shallo, Ericson, & R, 2002). During the alkaline extraction step, both phytic acid and globulins are solubilised. It is hypothesised that insoluble phytic acid-globulin aggregates are formed upon isoelectric point precipitation (Pei et al., 2019; Yong et al., 2021).

3.4. Drying step

The drying step can have a major impact on the protein's aggregated state, especially for spray drying, which is sometimes combined with a heating step to ensure microbial stability of the powdered ingredient. The spray-drying process can be tuned to avoid extensive aggregation and sometimes improve solubility and functional properties (Lili et al., 2015; Q. Zhao et al., 2013). We will not further elaborate on the impact of the drying process, as it an extensive topic has received attention, especially for animal-based proteins (Schuck, 2014). A sidenote here is the use of freeze-drying in academic studies, while spray-drying is a common method on industrial scale, which may reduce the translatability of academic studies to industrial application. Spray-dried plant-based protein ingredients should receive more attention in academia, especially on the type of aggregates formed and the impact on functionality.

3.5. Summary on the alkaline extraction-acid precipitation method

The conventional alkaline extraction-acid precipitation is commonly used by the food industry and in academic research to extract and fractionate plant proteins. The advantage is the effective removal of non-proteinaceous components, leading to a protein fraction with high protein purity. However, several drawbacks are present.

- The industrial defatting process induces protein denaturation, aggregation and oxidation.
- At high alkaline extraction pH, protein-phenol interactions are induced, leading to protein structure alteration and aggregation.
- The acid precipitation step can induce irreversible globulin aggregation.
- In the acid precipitation step, albumins, which can comprise up to 40 wt% of the proteins in the seeds, remain soluble and are often discarded. So, fractions produced using this method mainly contain globulins.

Finally, even though not further discussed in this work; the conventional method is energy- and resource-intensive with the generation of large side-streams. Such energy-intensive processing can reduce the sustainability aspect of plant protein fractions, as confirmed through a life cycle assessment (Lie-Piang, Braconi, Boom, & van der Padt, 2021).

4. Plant protein techno-functional properties

As described in the previous section, specific processing steps of the conventional process may immensely impact the protein's aggregated state. In the following section, we will evaluate how these aggregated structures affect the meso- and macroscopic properties of foams, emulsions and gels. To fully understand the underlying mechanism, each of following sections will start with a brief introduction on the key elements to achieve proper foaming, emulsifying or gelling properties, thus explaining the impact of the protein aggregates.

4.1. Air-water interfaces and foams

Foams are multiphase systems with a gas phase (e.g. air, N_2 or CO_2) dispersed in a liquid phase. Such systems can only exist due to the effective stabilisation of air bubbles or, to be more specific, the stabilisation of the air-water interface by, for instance, amphiphilic proteins (Amagliani et al., 2021). Two interfacial properties are required for air-water interface stabilisation: (1) The ability of the surface-active component to rapidly adsorb at the interface and reduce interfacial tension, for short-term stabilisation, and (2) subsequent interfacial interactions between the adsorbed proteins, leading to a stiff interfacial film (Narsimhan & Xiang, 2018). Protein adsorption reduces the interfacial tension and generally results in foams with small air bubbles with a narrow size distribution. This reduces the rate of disproportionation of air bubbles (i.e. diffusion of gas due to Laplace pressure differences), thus leading to increased foam stability (Foegeding, Luck, & Davis, 2006). A stiff interfacial film around the air bubble also reduces the rate of disproportionation. In addition, it reduces the chance of coalescence, where film rupture leads to the merging of air bubbles. Generally, soluble, relatively small, more mobile proteins possess excellent foaming and emulsifying properties (Mitropoulos, Mütze, & Fischer, 2014; F. Wang, Zhang, Xu, & Ma, 2020; M. Zhao, Xiong, Chen, Zhu, & Wang, 2020), such as dairy and egg proteins with a Mw of e.g. ~18 kDa for β-lactoglobulin (often exist as dimer of ~36 kDa) from milk and ~43 kDa for ovalbumin from eggs (Butré, Wierenga, & Gruppen, 2012; Wierenga, Meinders, Egmond, Voragen, & Jongh, 2003; J. Yang, Kornet, et al., 2022). In general, two main foaming properties are studied for protein-stabilised foams: (1) foamability (or capacity), the amount of foam that can be formed by the proteins, and (2) foam stability, which is often expressed as the time at which the foam volume decays by a certain percentage.

Solubility plays a major role in foam stabilisation. Insoluble proteins, often formed by aggregation, cannot diffuse rapidly to the interface, or form stiff interfacial layers and thus do not contribute to foam formation and stabilisation, and may even destabilise the foam (Amagliani et al., 2021; Moll, Salminen, Griesshaber, & Schmitt, 2022). Conventionally-processed plant protein extracts are abundant in highly aggregated globulin proteins, which show poor foamability and foam stabilising properties due to their low solubility and large aggregated structures (>300 kDa) (Ghumman, Kaur, & Singh, 2016; Wong et al., 2013; J. Yang, Mocking-Bode, et al., 2022). The insoluble globulin-based aggregates could be the result of irreversible aggregation during the industrial heat solvent extraction step or the isoelectric point precipitation. Another possibility for aggregation is the phenol-protein covalent interactions during the alkaline extraction step, thereby reducing foam stability (Rodríguez, von Staszewski, & Pilosof, 2015; J. Yang, Lamochi Roozalipour, et al., 2021).

4.2. Oil-water interfaces and emulsions

Emulsions consist of two immiscible liquids, of which one is dispersed in the other as droplets, forming oil-in-water (O/W) or water-in-oil (W/O) systems. To physically stabilise emulsions, emulsifiers are added, of which proteins are prominent representatives for food applications (Dickinson, 2011). Emulsion droplets with a diameter smaller than typically 1 μm are stable against creaming/sedimentation as induced by the density difference between the dispersed and continuous phases. When oil droplets flocculate, their effective size increases, making them prone to creaming or sedimentation. The same holds for larger droplets formed through coalescence, which may ultimately lead to oiling off. Whether these two latter effects occur depends mainly on how effectively the oil-water interface is stabilised.

Droplet break-up during emulsification is facilitated by a low interfacial tension. If not rapidly stabilised, the newly formed droplets immediately re-coalesce, leading to emulsions with larger and polydisperse droplet sizes (Tcholakova, Denkov, & Lips, 2008). Therefore, it is important that proteins quickly adsorb to the interface to reduce the interfacial tension and form an interfacial layer that stabilises the droplets. At pH values far away from the isoelectric point, protein-stabilised emulsion droplets are protected against flocculation

due to electrostatic repulsion, whereas at pH values close to the isoelectric point, emulsions may become unstable, because attractive (hydrophobic) droplet-droplet interactions predominate. Furthermore, the adsorbed protein layer provides a steric repulsion, which can prevent droplets from approaching close enough to coalesce. When both electrostatic and steric repulsions are not strong enough, droplets can come into close contact. At this point, the viscoelastic interfacial layer may still prevent coalescence.

Highly aggregated plant proteins could impact emulsifying properties, as shown for soybean proteins, where an extract with large insoluble protein structures (obtained using acid precipitation) formed less stable emulsions than those formed using smaller soybean protein aggregates (obtained by avoiding acid precipitation) (Kim & Kim, 2015; Rao et al., 2002). Similar behaviour was found for commercial pea protein-stabilised emulsions (Hinderink, Sagis, Schroën, & Berton-Carabin, 2020). The highly aggregated pea proteins could not adsorb rapidly onto the droplet's interface and stabilise it during emulsification, thus allowing re-coalescence of the oil droplets, leading to large oil droplets (Geerts, Nikiforidis, van der Goot, & van der Padt, 2017).

On the side note, the insoluble protein structures could posses high emulsion stability under specific conditions, forming particle-stabilised emulsions, also known as Pickering emulsions (Dickinson, 2020; Sarkar & Dickinson, 2020). The insoluble part of a commercial pea protein fraction could physically stabilise high internal phase emulsions over 14 days, giving ~10 times higher viscosity compared to whey protein-stabilised ones. In contrast, when using the full fraction (soluble + insoluble proteins) of the commercial isolate, emulsions were physically unstable and coalesced over time (Hinderink, Schröder, Sagis, Schroën, & Berton-Carabin, 2021). Having a pure plant protein particle system seems to be crucial in obtaining stable oil droplets.

4.3. Gelation

Gels are semi-solid systems, mainly comprised of liquid entrapped in a three-dimensional cross-linked network. Proteins can form gels due to their intermolecular interactions. The system can be considered a gel once the percolation threshold is reached; a situation in which the degree of intermolecular linking has led to a space-spanning network (Hinderink, Boire, et al., 2021). There are a variety of methods to induce gelation, including heat-, acid-, chemically- or enzymatically-induced gelation. In this review, we will focus on the heat-induced gelation of plant proteins.

Heat-induced gelation occurs via several steps. The first step is the unfolding or dissociation of proteins (e.g. heat-induced protein denaturation), which is followed by a second step; aggregation of the unfolded proteins due to interactions between newly exposed hydrophobic groups, and in some cases disulphide-bridge formation, to form aggregates. These are referred to as 'primary' aggregates, and a second aggregation step is required, where the primary aggregates form a space-filling gel network (Totosaus, Montejano, Salazar, & Guerrero, 2002). Globular proteins have buried hydrophobic regions, which are required to interact upon unfolding for effective primary aggregate and subsequent three-dimensional network formation. Here, several protein properties/parameters affect the gelation: (1) The protein concentration, as a sufficient amount of protein is required to form a space-spanning network; (2). The protein charge, as the electrostatic repulsion affects the network formation (Nicolai, Britten, & Schmitt, 2011); (3) The protein solubility, as high protein solubility results in macroscopically homogeneous gels (Nicolai & Chassenieux, 2019); (4) The proteins aggregated state determining the availability of the hydrophobic regions, especially the latter will be carefully discussed in the following paragraphs.

Globulin proteins are globular and should possess the ability to form gel networks. Especially in a native state with high solubility, the proteins are homogeneously distributed in the solution. As a result, the well-distributed proteins can denature upon heating and form primary aggregates, followed by secondary aggregation into a space-spanning homogenous gel network (Kornet, Veenemans, et al., 2021). However, process-induced aggregated proteins would not be able to form such gel, often due to lower solubility and poor distribution in the system. This pre-aggregation could lead to heterogeneous gel structures. Works on yellow pea, soy, lentil and faba bean showed that extensive pre-aggregation of protein prior to the actual gelation step (due to acid precipitation) seems to give weaker and more brittle gels, as the proteins are now embedded in the pre-aggregated structures and have fewer interaction sites per unit of volume compared to native proteins (Alonso et al., 2019; Kornet, Veenemans, et al., 2021; Langton et al., 2020; Monteiro & Lopes, 2019). The conventional processing method of the plant protein fractions precipitation and heating steps may lead to extensive protein denaturation and the formation of pre-aggregates. As a result, a higher protein content of such non-soluble, highly denatured and aggregated plant proteins is required compared to less aggregated ones to fill the space (Kornet, Veenemans, et al., 2021; Q. Yang, Kornet, et al., 2022).

5. Preventing protein aggregation to improve functionality using process steps

It is evident that formation of large (and insoluble) aggregates may heavily impair techno-functionality. Therefore, in the following sections, we will focus on obtaining more functional proteins by changing specific processing steps that would normally induce protein aggregation, which are isoelectric point precipitation and heating during oil extraction.

5.1. Replacing isoelectric point precipitation with membrane filtration

The formation of irreversible protein aggregates by isoelectric point precipitation can be omitted as was shown for purification of lupin and pea protein fractions (Berghout, Boom, & Van Der Goot, 2014; Geerts, Mienis, Nikiforidis, van der Padt, & van der Goot, 2017; Kornet, Veenemans, et al., 2021). The starch-rich lupin and pea seeds were milled, alkaline wet extracted, centrifuged to remove insoluble components, and finally (spray-)dried. These protein extracts contain both (partly) native globulins and albumins (the latter are normally removed by the acid precipitation step). By omitting the acid precipitation step, protein aggregation was prevented, resulting in protein fractions with a high solubility (>90%, at room temperature) (Berghout et al., 2014; Kornet et al., 2020).

The consequence of omitting the acid precipitation step is that soluble non-proteinaceous components (i.e. phenols and anti-nutritional components) remain in the protein-rich solution, as shown for a yellow pea protein extract obtained by alkaline extraction at pH 8, followed by centrifugation and drying of the supernatant, with 49% non-proteinaceous components, while a conventional extract only had 13% of those components (Kornet, Veenemans, et al., 2021). These are generally low molecular weight components (<5 kDa), and can be removed using membrane filtration, also known as ultrafiltration in this context and can be combined with diafiltration (Alonso et al., 2019). Ultrafiltration is a separation process in which small molecules (e.g. sugars, salts) are forced through a semipermeable membrane using pressure or concentration gradients. This results in a retentate depleted of small molecules and purified in protein (>5 kDa). In the case of diafiltration, the retentate is recirculated and diluted with a buffer or water, to further remove salts in the purified protein solution.

Membrane filtration was shown to be effective in remove low molecular weight components for pea (Kornet, Veenemans, et al., 2021), rapeseed (Ntone, Bitter, & Nikiforidis, 2020) and Bambara groundnut (J. Yang, de Wit, et al., 2022) protein fractions. For pea, a filtrated fraction with a protein purity of 88% could be obtained (Kornet, Veenemans, et al., 2021). Filtration also leads to protein fractions with lighter colours

for rapeseed and soybean protein fractions (Kim & Kim, 2015; Ntone et al., 2020), due to removal of phenols and tannins. In addition, preventing covalent protein-phenol interactions produces less altered protein structures and protein-phenol aggregates (Keppler et al., 2020). Another reason to filtrate is the removal of minerals which could impact the local charges of protein molecules (Salgin, Salgin, & Bahadir, 2012). Finally, membrane filtration could reduce off-flavours in the final protein extract, as for instance shown for membrane-filtrated soy protein extracts (<150 ppb) (Damodaran & Arora, 2013).

The presence of more native globulin proteins showed an immense improvement in techno-functional properties. Mild purification of proteins from lentil, yellow pea and Bambara groundnut seems promising to increase their foaming properties, compared to protein fractions obtained using the conventional method (Alonso et al., 2019; J. Yang et al., 2020; J. Yang, de Wit, et al., 2022). Here, the more native proteins were able to adsorb faster at the air-water interface and forming stiffer protein layers, leading to improved foaming. Furthermore, the presence of albumins seems to play a foam improving role as well. This was, for instance, shown for mildly fractionated yellow pea and quinoa protein extracts containing both albumins and globulins (Van de Vondel, Janssen, Wouters, & Delcour, 2023; J. Yang, Mocking-Bode, et al., 2022). Here, albumins and globulins co-adsorbed at the air-water interface at pH 7.0. At pH (3.8–5.0), the globulin proteins were mostly in solution, while albumins seemed to dominate the interface, giving high foamability and stability. For rapeseed protein-stabilised foams, native albumins and globulins were found to have a synergistic effect, where the albumin allowed the formation of small air bubbles and high foam volumes, while the globulins assisted by increasing interfacial stiffness, thus leading to the creation of a stable foam (P. Shen, Yang, Nikiforidis, Mocking-Bode, & Sagis, 2023). This previously mentioned work is thus showing the potential of globulin proteins in stabilising air-water interfaces and foams, if extracted in a native way, thus allowing the protein's molecular properties to directly contribute to its techno-functionality.

These plant albumins show foaming properties similar to those of dairy and egg proteins (J. Yang, Kornet, et al., 2022). The underlying reason could be the small albumin size (10–53 kDa). Furthermore, albumins have a lower net protein charge (at neutral pH) compared to globulins (Ghumman et al., 2016; J. Yang, Kornet, et al., 2022). The small size and low charge lead to a higher surface coverage by albumins than globulins, as smaller proteins give a more effective packing and a lower charge allows the proteins to approach each other more on the surface. This may lead to smaller air bubbles with stiffer interfaces and higher foam stability.

Pea protein extracts obtained with and without acid precipitation were compared for their emulsion properties. Omitting acid precipitation led to a substantially stiffer oil-water interface, which probably prevented re-coalescence during homogenisation. As a result, five times smaller oil droplets were formed compared to the acid precipitation-obtained pea protein extract (Geerts, Nikiforidis, et al., 2017). The type of interface formed by pea proteins with aggregated state was also studied using front-surface fluorescence, where the tertiary protein structures before and after adsorption was measured. Commercial pea proteins (obtained with acid precipitation) were present as soluble aggregates and adsorbed as such at the interface. Less-aggregated pea proteins (in-house produced using acid precipitation) adsorbed with the tryptophan-free region at the oil-water interface, suggesting that the less aggregated protein structures were less rigid than the large protein aggregates in the commercial isolate (Hinderink, Berton-Carabin, et al., 2021). These findings show the importance of avoiding aggregate formation during the extraction and fractionation processes for emulsions.

Similar to the foaming properties, the albumins that are preserved by omitting acid precipitation can influence the emulsion stability. When comparing pea protein albumin- or globulin-stabilised emulsions, globulin-stabilised emulsions had a higher stability against flocculation at pH 7.0. This can be explained by their higher charge resulting in electrostatic repulsion (Kornet, Yang, Venema, van der Linden, & Sagis, 2022). In a mixture, both proteins are likely to be present at the interface, as shown for pea and rapeseed emulsions. Here, the globulin proteins seem to dominate the stability of the system, mostly by providing a high surface charge on the droplet (Kornet et al., 2022; Ntone et al., 2021).

For gelation, avoiding the pre-aggregation after acid precipitation is crucial in obtaining homogeneous and stiff gels. Yellow pea protein ingredients in a native state (due to omitting acid precipitation) formed stiffer gels at lower concentrations than proteins obtained using acid precipitation (Kornet, Penris, et al., 2021). The same two types of protein ingredients from faba beans and lentil also showed the formation of stiffer gels by more native and less aggregated protein fraction, with a lower least gelation concentration (Alonso et al., 2019; Langton et al., 2020). A side note here is that not all structuring applications require native and high protein solubility, where an example is meat analogues, where protein isolates with a low solubility may be useful to create anisotropic structures in high-moisture extrusion (Wittek, Zeiler, Karbstein, & Emin, 2021). Geerts et al. demonstrated how native soy proteins had low water-holding capacity and were unable to form fibrous structures under shear. Here, the toasting of the soy proteins (i.e. inducing aggregation) led to the increased ability to form fibrous structures (Geerts, Dekkers, van der Padt, & van der Goot, 2018).

5.2. Using neutral pH conditions during extraction

Extreme alkaline conditions are sometimes used in plant protein extraction, and as mentioned in section 3.2, a lurking risk is protein-phenol interactions, resulting in darker colours, off-flavours and large protein-phenol aggregates. Here, the type of protein-phenol interaction seems to largely affect its impact on techno-functionality. The most significant negative impact on techno-functionality was found for covalently-bonded protein-phenol aggregates, which reduced foam stability, due to weaker interfacial films, and lower gel strength, due to pre-mature aggregation (Chen et al., 2021; Rodríguez et al., 2015; J. Yang, Lamochi Roozalipour, et al., 2021).

An optimal extraction pH value, for each source, can prevent the irreversible complexation of proteins with the co-extracted phenols, while ensuring high protein extraction yields. For example, sunflower seed should be extracted at a pH below 8.0 to decrease the rate of sunflower protein-chlorogenic acid (major phenol in sunflower seed) complexation (Karefyllakis et al., 2018), while the extraction pH of rapeseed should be maintained at values below 9.0 to avoid the oxidation of sinapic acid (Cai et al., 1999; Ntone et al., 2020; Xu & Diosady, 2000).

While covalent interactions will be prevented, noncovalent interactions between phenols and proteins may still occur at a neutral pH, which could alter the protein (2D and 3D) structure and surface properties (charge and hydrophobicity). The lower surface hydrophobicity of proteins upon noncovalent protein binding on the protein's hydrophobic surface patches may increase protein solubility, leading to higher foamability, as shown for sunflower, soy and pea proteins (Hao et al., 2022; Jiang, Zhang, Zhao, & Liu, 2018; Subaşı et al., 2020; Sui et al., 2018). Phenols might also exist as unbound phenols interfering with protein functionality. For example, phenols compete with proteins at the air-water interface, leading to less stable foams; flocculated oil droplets; and crosslinking proteins upon gelation, leading to stiffer gels (Ntone, Qu, et al., 2022; Strauss & Gibson, 2004; J. Yang, Lamochi Roozalipour, et al., 2021).

5.3. Omitting oil extraction

Defatting, at high temperatures and using organic solvents, is one of the major protein-altering steps in conventional extraction process (Berghout, Pelgrom, Schutyser, Boom, & Van Der Goot, 2015). A milder alternative is cold-pressing of oilseeds, where the seeds are directly

processed in a mechanical press, giving a short exposure to elevated temperatures of 50–60 °C (Carre, 2021; Leming & Lember, 2005). The protein-rich meal from cold-pressed oilseed contains less denatured and aggregated proteins (Fetzer et al., 2018). Overall, oil extraction yields are comparable to those obtained using conventional defatting processes (Carre, 2021; Fetzer et al., 2018). A major advantage is the fact that the residual oil present in the protein-rich meal (from cold-pressing) is present in its natural state, namely oleosomes (Karefyllakis, Octaviana, van der Goot, & Nikiforidis, 2019).

The conventional defatting (pressing and solvent extraction) and milling step can be completely omitted, which is shown for rapeseed and Bambara groundnut. In this processes, dehulled seeds were soaked at alkaline pH of 9.0–9.5, and seeds were disrupted by blending and twin-screw pressing (Ntone et al., 2020; J. Yang, de Wit, et al., 2022). This process resulted in the co-extraction of proteins and intact oleosomes. Oleosomes are dispersible in water at these conditions, as the surface is hydrophilic, and negatively charged at alkaline conditions (Nikiforidis, 2019). Subsequently, oleosomes and proteins can be separated using centrifugation, which yields a pellet with insoluble components, a protein-rich middle layer, and an oleosome-rich cream layer (Ntone et al., 2020). This cream has the potential to be used in foods as natural emulsions (Ntone et al., 2023). The middle protein-rich layer can be further processed by membrane filtration and drying to yield a fraction with 11–15 wt% oil content (in the form of oleosomes) and 62–65 wt% protein (Ntone et al., 2020; J. Yang, de Wit, et al., 2022). This protein extract can have protein yield values of 77–79% and possess substantially more native and less aggregated proteins compared to an protein extract obtained using conventional defatting and acid precipitation (Lie-Piang et al., 2023; Ntone et al., 2020).

A drawback here could be the presence of lipids, which may especially impact foaming properties. Surface-active lipids (i.e. phospholipids) compete with proteins for adsorption at the interface and may displace adsorbed proteins (Rodríguez Patino, Carrera Sánchez, & Rodríguez Niño, 2008). This reduces the interfacial connectivity of proteins thus decreasing film strength, foamability and foam stability (Wilde et al., 2003). Lipid droplets, such as oleosomes, have an anti-foaming mechanism by bridging the interfacial layers of two neighbouring air bubbles, which can induce film rupture (Denkov & Marinova, 2006). While for foaming applications, the foam destabilising properties of oleosomes are undesired, it might be suitable as an anti-foam ingredient, which reduces foam formation upon protein extraction, as the anti-foaming properties of oleosomes can be controlled by changing the protein-to-oleosome ratio (J. Yang, Berton-Carabin, Nikiforidis, van der Linden, & Sagis, 2021).

Lipids in the form of oleosomes could be removed using filtration, yielding protein extracts that are high in native proteins, which could form high and stable foam volumes, stable emulsions and form stiff gels with a homogenous microstructure (Ntone et al., 2021; Ntone, Kornet, et al., 2022; J. Yang et al., 2020). Alternatively, the oleosome-containing protein extract can be used as such, which could be used to form oil-filled gels (Ntone, Kornet, et al., 2022). In emulsion applications, the oleosomes can act as colloidal emulsifier, as they can adsorb and stabilise free oil (Ntone et al., 2023).

A final point of attention is lipid oxidation, as the lipids are prone to oxidation, which negatively affects the sensory characteristics of the ingredients (Sharan et al., 2022). Some authors even state that lipid oxidation in the main mechanism of flavour instabilities in plant protein ingredients (Mehle, Paravisini, & Peterson, 2020). Lipoxygenase is associated with the main oxidative pathway. In the seeds and during downstream processing, lipoxygenases and fatty acids in proximity, which enhances the oxidative destabilisation (Liu, Cadwallader, & Drake, 2023). Further understanding and control of lipid oxidation in the protein ingredients is required for their successful application in food products (Keuleyan et al., 2023).

5.4. Mild protein extraction and fractionation

We have now discussed the impact of alternative processing steps on proteins techno-functional properties focussing on (1) omitting acid precipitation, (2) extraction pH, (3) omitting oil extraction. Using one of or combining these new process steps can be considered an alternative process, which is known as mild (or gentle) extraction and fractionation processing. Examples for a wet mild process for starch-rich or oil-rich seeds are shown in Fig. 4, where in both cases acid precipitation is replaced by membrane filtration, and the defatting process is fully omitted for the oil-rich seeds. Albumin proteins remain present in the main fraction, when using mild fractionation. While the protein content of mildly-fractionated protein ingredients could be lower than that of conventionally-fractionated ones, the fewer fractionation steps and albumin retention could lead to higher protein yields (Loveday, 2020). The term ‘mild’ could also refer to the amount of used resources, as mild processes could use fewer processing steps, use lower amounts of energy, heat and extreme pH conditions and water, and produce fewer side-streams and losses compared to the conventional process (Lie-Piang et al., 2021).

In summary, mild alternatives are available to overcome the drawbacks of conventional wet fractionation. The methods omit, replace or tune specific steps of the conventional process. One risk that should receive urgent attention is the co-extraction of non-proteinaceous components, such could impair techno-functionality, but also impact the sensory or nutritional aspect. One may argue that minor changes in existing processes would be favourable, since fewer process alterations are needed which makes implementation faster and easier. However, others have proposed new and more diverse mild fractionation methods, such as the use of electrophoretic mobility-based separation and dry fractionation. We refer to several recent papers on these topics (Fritz, Bera, et al., 2021; Fritz, Boom, & Schroën, 2021; Lie-Piang et al., 2023)

5.5. Post-fractionation processing

The presence of large process-induced aggregated structures in plant protein ingredients receives increasing attention in studies. While in our opinion, avoiding process-induced aggregation during extraction/fractionation (as this is an inevitable process) would be most favourable and energy efficient, there is current focus on breaking down the large and insoluble aggregates using post-fractionation processing, with the primary focus to increase solubility. Recent reviews elaborate on physical, chemical and biological post-fractionation processes that may increase protein solubility (Grossmann & McClements, 2023; Nikbakht Nasrabadi, Sedaghat Doost, & Mezzenga, 2021).

The physical processes are especially focused on breaking the large aggregated protein structures into smaller more soluble structures. Examples are ultrasonication, pulse electric field, and high-pressure homogenisation. High-pressure homogenisation has a high potential for applications, as homogenisers are usually widely available in industry. For commercial pea and lupin protein isolates, it has been shown that large aggregated structures can be (partly) broken down during high-pressure homogenisation (Keuleyan et al., 2023). Another work showed similar results for pea and faba bean proteins showed a similar result, which was linked to higher solubility and improved emulsion and foaming stability (D’Alessio et al., 2023; J. Yang, Liu, Zeng, & Chen, 2018). High pressure homogenisation of insoluble large structures simply results in more ‘soluble aggregates’, and not per se single native protein structures. These soluble aggregates are expected to behave differently than mildly purified ingredients where the naturally-present native state is as much as possible retained.

6. Summary and future outlook

The search for functional plant-based protein ingredients is rapidly expanding, with the focus on obtaining protein fractions with high

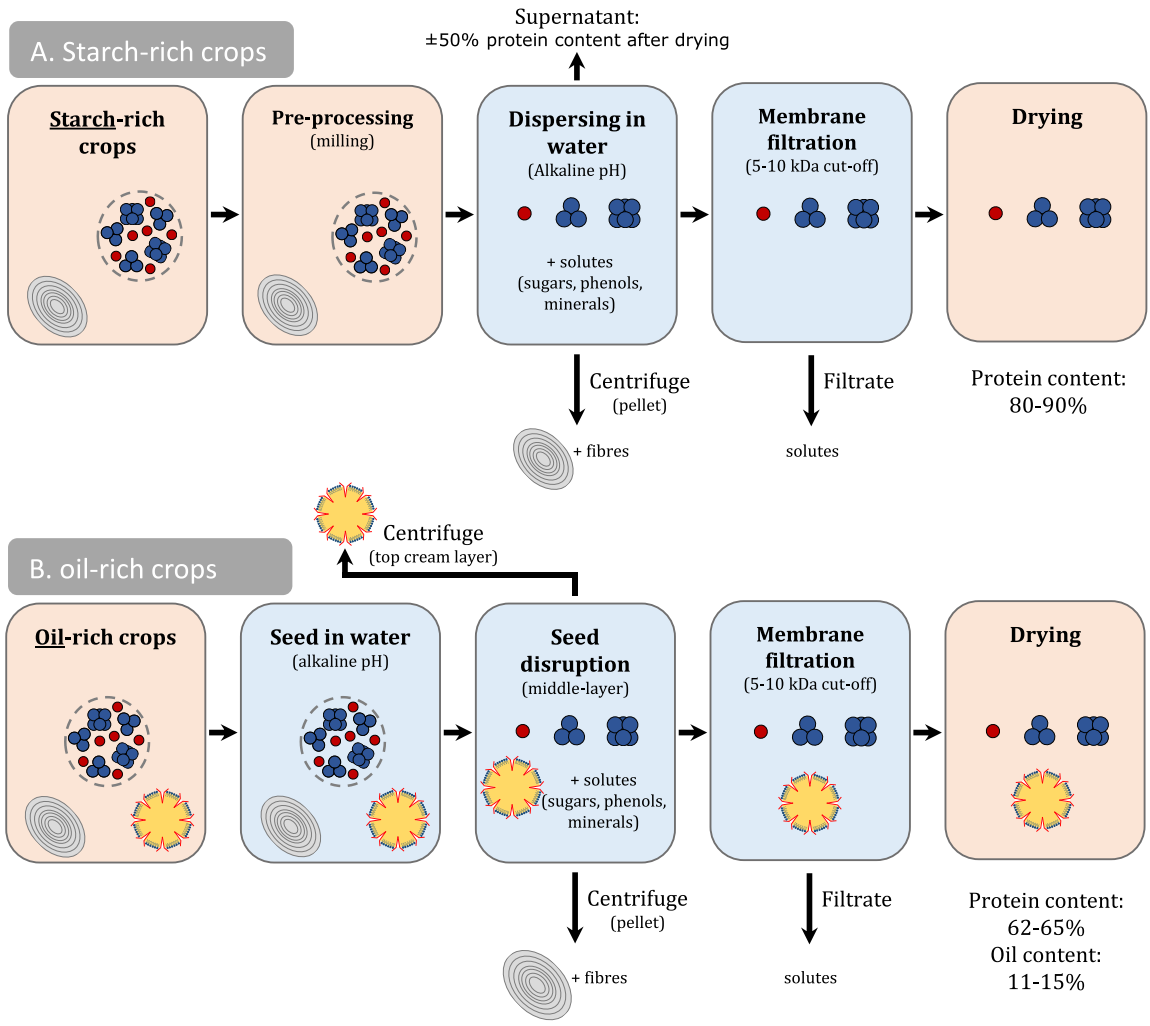

Fig. 4. Schematic overview of the mild wet protein fractionation methods.

purity. Here, the process history is often not taken into account. Since it has an undeniable impact on protein functionality we discussed the impact of fractionation steps on the proteins functional properties.

We have shown how specific protein extraction or fractionation steps can aggregate proteins into large, insoluble aggregated structures. Especially the conventional wet extraction method can largely increase the aggregate size by extensive heat denaturation upon defatting, protein-phenol interactions and irreversible aggregation after acid precipitation. We have discussed how these large aggregates negatively affect the foaming, emulsifying and gelling properties. The formation of such large structures seems to dominate the functional properties rather than single protein molecular parameters such as charge of the amino-acids and hydrophobicity.

Currently, there is increasing focus in the field on the post-fractionation processing, which uses physical processes such as high-pressure homogenisation to break-down these large aggregated protein structures to smaller ones with sometimes enhanced solubility and functionality. However, we urge to prevent aggregation in the current extraction and fractionation processes. The foaming, emulsifying and gelling properties of plant proteins generally improve when more native proteins are extracted. Such mild/gentle processing could really assist in obtaining functional protein fractions with potentially higher protein yields and lower use of resources. Furthermore, by preserving the native state during processing, the protein composition, structure, and surface properties might again play a bigger role in techno-functionality.

A main attention point for further research is the co-extraction of non-proteinaceous components, as mild extraction could lead to less pure protein ingredients, thus more impurities. While functionality has received increasing attention, the sensory and nutritional aspects should definitely be a key focus point in future studies.

Finally, by better understanding the role of processing on functionality, we could achieve a functionality-driven manner of protein extraction and fractionation. Here, plant proteins are not purely extracted based on protein yield, but also on the aggregated state, thus functionality, in the final ingredient. We believe such a functionality-driven protein extraction/fractionation is key in securing a stable food system for the near future.

CRediT authorship contribution statement

Jack Yang: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Remco Kornet:** Writing – original draft. **Eleni Ntone:** Writing – original draft. **Maud G. J. Meijers:** Writing – original draft. **Irene A.F. van den Hoek:** Writing – original draft. **Leonard M.C. Sagis:** Writing – review & editing. **Paul Venema:** Writing – review & editing. **Marcel B.J. Meinders:** Writing – review & editing, Supervision. **Claire C. Berton-Carabin:** Writing – review & editing. **Constantinos V. Nikiforidis:** Writing – review & editing. **Emma B.A. Hinderink:** Writing – original draft, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have declared that no competing interest exist.

Data availability

Data will be made available on request.

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