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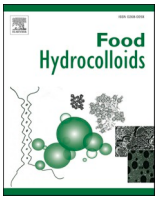

The generality of pH-induced liquid-liquid phase separation in plant proteins extends to commercial legume flours

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ABSTRACT

This study explores the phenomenon of liquid-liquid phase separation in protein-containing dispersions from commercial soybean, yellow pea, and fava bean flours. Phase separation in these cases is also referred to as simple coacervation. The phenomenon is shown to occur for any of these legume sources. Flours were dispersed at alkaline conditions at concentrations ranging from 15 %w/w – 21 %w/w, insoluble fractions were removed, and the soluble fractions were slowly acidified. For all flours, we observe three characteristic pH ranges using microscopic analysis. A high pH range of soluble proteins, an intermediate pH range in which microscopic (roughly spherical) protein particles are formed (so-called coacervation range), and a low pH range in which the spherical protein particles cluster and create larger scale structures. Particle size distribution measurements confirmed these observations, allowing the boundaries between the different pH regions to be delineated more precisely. We find some, but contrary to other studies, no extensive fractionation of proteins of distinct types over the spherical protein particles and the continuous phase in the pH range of coacervation. Our work points to the generality of liquid-liquid phase separation in flours of legumes.

We demonstrate that simple acidification can induce phase separation across different legume protein mixtures, offering a generalisable method not restricted by the conditions of specific protein fractions. Unlike conventional salt-induced methods, our approach requires no additional salt or purification, maintaining the product's suitability for food applications.

1. Introduction

Substantial efforts are underway to produce new plant-based food products, among others, as substitutes for animal-derived foods such as meat and dairy. These novel food products could facilitate and contribute to transitioning from an animal-based diet to a more sustainable plant-based alternative for a sizeable portion of the population (Beverland, 2014).

Developing plant-based alternatives for dairy and meat presents immediate challenges, particularly in accurately replicating their sensory properties and physicochemical characteristics. A major hurdle is the limited structuring ability of commercial plant protein ingredients. To address this, current plant-based products often combine plant proteins with non-protein structuring agents such as methylcellulose, gums, and starch to compensate for their inadequate functionality. This underscores the complexity of the task and the need for innovative solutions in food science (Bakhsh et al., 2021; Grossmann & Mcclements, 2021; Mattice & Marangoni, 2020).

Therefore, a crucial challenge in developing plant-based alternatives to animal-based products is to improve plant protein ingredients' structuring ability so that non-protein-structuring ingredients that serve as merely structuring additives can be eliminated. In an ideal case, the plant proteins should provide good structuring functionalities, like animal-based proteins like whey proteins in set yoghurt, which form a stable protein-continuous network (Modler & Kalab, 1983; Puvanenthiran et al., 2002), or like the casein in processed cheese, which entraps fat droplets and forms a stable protein-continuous network (Green et al., 1986; Marchesseau et al., 1997).

From a structuring point of view, the challenge for using plant protein ingredients in formulating plant-based foods is to balance sustainability, protein functionality, and protein content. Protein solutions from legume flours and their concentrates contain a low protein content of 20 %w/w—27 %w/w and 64 %w/w—86 %w/w, respectively. The proteins are in a relatively native state and thus effective in

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physicochemical functionality (Ma et al.). This effectivity is better than that of their so-called isolate counterparts, which contain higher protein content, i.e., 75 %w/w – 96 %w/w, and the harsh processing conditions often cause this during the production of the isolates (Schutyser et al., 2015). Such harsh processing also makes them less preferred from a sustainability point of view (Sim et al., 2021).

New and improved milling methods are among the approaches to extracting and processing plant proteins with enhanced functionality while keeping costs competitive (Palavecino et al., 2019). New dry (Assatory et al., 2019; Schutyser, 2011) and wet extraction methods (Stock et al., 1999), and alternative procedures, such as mild hybrid separation (Yang et al., 2022) are also being investigated. These technological advancements aim to develop high-quality plant protein ingredients that meet the food industry's requirements for functional, sustainable, and cost-effective ingredients with good sensorial properties.

Despite significant scientific advancements, wet extraction using isoelectric precipitation (IEP) remains the most widely used method for extracting proteins from plant materials. In IEP, the pH is adjusted to around the isoelectric point of the proteins, resulting in decreased solubility and protein precipitation (Petenate & Glatz, 1983). An alternative method, salt extraction (SE), works by adding salt to the soluble plant material, causing protein precipitation and subsequent separation (Karaca et al., 2011). Even though SE is thought to better preserve the proteins' food functionality, IEP is still often preferred due to its lower cost. In IEP, precipitation is followed by spray drying the protein solution to produce a so-called isolate powder.

Early research investigated more modest pH reductions to pH values slightly above the protein isoelectric point as a variant of IEP. Early work on purified Arachin, a groundnut protein, showed that this may lead to the formation of highly concentrated, spherical, micron-sized liquid protein droplets rather than solid-like protein precipitates (Tombs et al., 1974). These phase-separated protein-rich droplets, resulting from liquid-liquid phase separation, could be advantageous for food structuring due to proteins' high structuring capacity. However, this promising line of research was not pursued further.

The droplet formation is thought to be due to liquid-liquid phase separation, which (for polymer and biopolymer solutions) is also known as coacervation. We recently conducted an in-depth review discussing the conditions for coacervation in plant proteins and their application in food processing (Doshi et al., 2024). The best-studied case of coacervation is the so-called complex coacervation, in which electrostatic attractions between oppositely charged biopolymers drive phase separation (De Kruif et al., 2004), such as gelatine and whey (Mohanty & Bohidar, 2005; Nicolai & Durand, 2013; Shimokawa et al., 2013) and plant proteins with other polysaccharides (Li et al., 2021). Such complex coacervation has been studied for decades and is used, among other things, for encapsulation (Balassa et al., 1971; Muthuselvi & Dhathathreyan, 2006; Tolle, 1966).

The phenomenon we are concerned with here is liquid-liquid phase separation arising from plant storage proteins (Lazko et al., 2004). The increasing demand for plant-based protein ingredients has led to renewed interest in plant protein coacervation as a means of structuring using plant proteins (Popello et al., 1991, 1992; Suchkov et al., 1997).

Simple coacervation is the phase separation process where a liquid polymer or protein solution forms two distinct liquid phases, with one being rich in the polymer (the coacervate) and the other being a dilute solvent phase, typically driven by changes in factors like pH, temperature, or salt concentration. Purified plant protein fractions such as soy and pea globulins have been studied recently for their potential to form simple coacervate droplets. Moderate increases in temperature, which are not enough to lead to protein aggregation, may lead to the disappearance of the liquid-liquid phase separation, and this opens interesting kinetic effects for the heat-set gelation of plant protein simple coacervates. Microgels filled with vacuoles or microcapsules may be formed depending on heating conditions (Chen et al., 2016a, 2016b, 2017). Other recent studies have explored the salt-induced coacervation in yellow pea protein (Kornet et al., 2022). and mung bean protein (Yang et al., 2023) to develop highly concentrated protein particles using heat-set gelation of simple coacervates. We can also create protein microgel structures that are claimed to improve sensory properties and lower astringency (Kew et al., 2023).

There is evidence that simple coacervation is a generic phenomenon for purified legume storage proteins. Still, more effort is needed to compare the coacervation of plant proteins in protein mixtures such as flour. Therefore, we aim to compare simple coacervation of proteins in different legume flours, focusing on food structuring applications.

Defatted soy flour, yellow pea flour, and fava bean flour are used as model systems. These systems have been previously studied to various degrees in purified fraction and salt-induced phase separation. Most literature studies add salt to induce liquid-liquid phase separation in these plant proteins. However, this approach results in a significantly high salt concentration in the resultant product, which raises concerns regarding the food product's overall acceptability.

The current investigation demonstrates that pH-shift-induced simple coacervation is a general phenomenon in leguminous proteins. We include soy as a reference case since simple coacervation from defatted soy flour (Lui et al., 2007) and purified soy proteins, such as glycinin (Chen et al., 2018, 2020a; Lazko et al., 2004) has been studied for various environmental conditions.

We include yellow pea, a key protein source commonly studied and used for plant-based product formulation. Recent studies have provided insights into the process of droplet formation and microcapsule formation from purified and freeze-dried protein isolates, and salt-induced phase separation has recently become available (Cochereau et al., 2019; Kornet et al., 2022). We also employ whole legume flours to avoid further purification and freeze-drying steps for pea protein isolation. Finally, we include fava bean as another protein source that is also gaining importance for formulating plant-based food products and has shown the potential for forming salt-induced hollow microcapsules using purified legumin fractions (Zhao et al., 2021).

We disperse the flour at alkaline pH to extract the soluble proteins while removing the insoluble material (mostly fibre and starch). Next, we apply a slow, stepwise pH reduction. This pH reduction reduces the electrostatic repulsion between the proteins to the extent that they experience a net weak mutual attraction, which causes them to form liquid droplets while no solid precipitates.

First, we focus on establishing pH boundaries for the protein coacervation process for different legume flours using (fluorescence) microscopy. Next, we analyse the changes upon acidification in particle diameter, dry matter content and protein solubility. We perform SDS-PAGE analysis on the various soy, yellow pea, and fava protein solutions under reducing conditions to investigate the extent of partitioning over the droplet and continuous phases by the different protein-specific fractions.

This study is novel in its focus on the general applicability of pH-shift-induced simple coacervation across multiple legume flours—defatted soy flour, yellow pea flour, and fava bean flour—as a

method for plant protein structuring. While most studies explore specific legume proteins (or protein fractions) in purified or salt-induced conditions, our research demonstrates that simple acidification can induce liquid-liquid phase separation without added salt or purification, yielding a practical, low-salt alternative suitable for food applications. This multi-component, whole-flour approach provides a generalisable method applicable across legume species, supporting sustainable food formulations.

2. Materials and methods

2.1. Materials

Defatted soy flour was obtained from Sigma Aldrich (Schnelldorf, Germany), and yellow pea flour and fava bean flour were obtained from Erbo ingredients (Wormer, The Netherlands). 1M NaOH and 1M H_2SO_4 were used to adjust the pH. Rhodamine B for CLSM was obtained from Sigma Aldrich (Schnelldorf, Germany), and MilliQ® water was used from Elga Veolia (High Wycombe, United Kingdom).

2.2. Sample preparation

Three sample concentrations (15 %w/w, 18 %w/w, and 21 %w/w) were dispersed in MilliQ® water. The sample pH was adjusted to pH 8.3 ± 0.5, as shown in Fig. 1, for each legume flour using 1M NaOH. The flour dispersions were stirred at room temperature (294 K ± 1K) for 2 h, and the pH was monitored and adjusted to maintain a pH of 8.3. Insoluble fractions such as fibres, starch, and insoluble proteins were removed by centrifugation (Thermo Scientific Sorvall Lynx 4000) at 17,000 RCF for 30 min at 294 K. The resulting supernatant was decanted, stored at 277 K, and used within four days of preparation. This is regarded as protein dispersions throughout the text. Samples were brought to room temperature before acidification. They were gradually acidified using 1 M H_2SO_4 to induce coacervation.

2.3. Laser diffraction particle size distribution

The particle diameter of the samples was determined using laser diffraction, where a Hydro SM and Hydro S unit of the Mastersizer 2000 (Malvern Instruments, United Kingdom) were used. We used deionised water at a stirring rate of 1700 RPM to disperse the samples with intermediate cleaning and background measurement steps. The samples' absorption and refractive indexes were set to 0.001 and 1.45, respectively (Kornet et al., 2022). The dispersant, Deionised water, was set to a refractive index of 1.33. We obtained the results in a volume-weighted distribution, which is biased towards larger particles because it considers their volume. D (0.5) was used as the median size distribution. All samples were performed at least triplicate measurements.

2.4. Confocal scanning laser microscopy (CLSM)

The 0.005 %w/w Rhodamine B stock was prepared in MilliQ® water. Proteins were stained by adding 5 ppm Rhodamine B to protein dispersion during the sample preparation step. This was followed by 2 h of stirring and acidification to reach the desired pH. 30 µl protein sample was pipetted on the microscopy slide.

An inverted CLSM Nikon Eclipse Ti2 was used to observe the samples. The samples were observed using a 100X Plan Apochromat silicon oil immersion objective with a 1.35 Numerical Aperture, #1.5 coverslip with 0.17 mm thickness, and correction on the objective's correction collar slider. The samples were excited using a 561 nm laser and detected using a 525/50 nm. Bright-field images were collected in a secondary channel. All images were processed using Fiji (Image J) software.

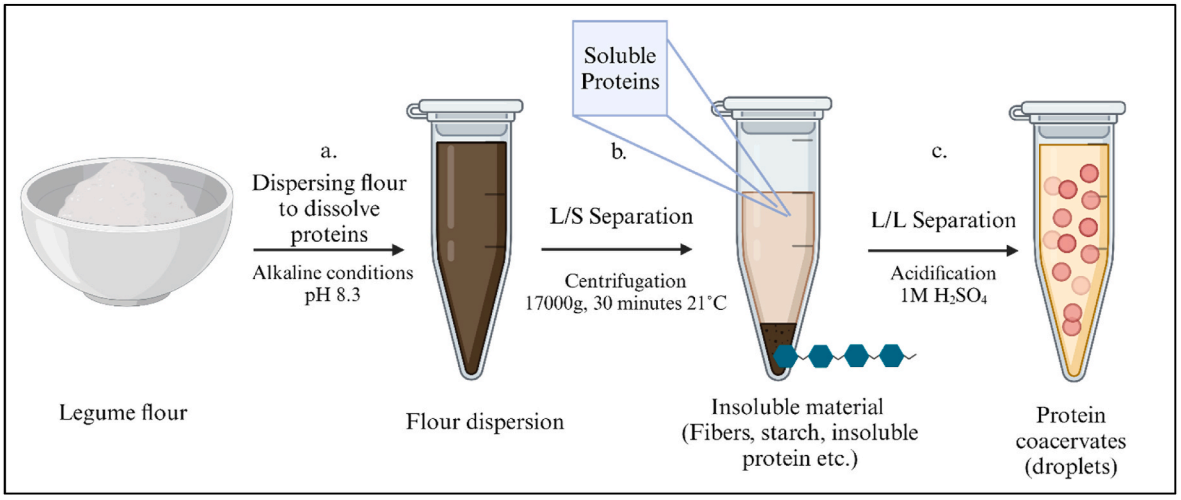

Fig. 1. Schematic procedure for inducing liquid-liquid phase separation (coacervation) using legume flour. (a) The flour is dispersed in alkaline conditions (pH 8.3). (b) Insoluble fractions such as fibers, starch and insoluble proteins are centrifuged out by liquid-solid separation. (c) The proteins are soluble in supernatant phase. A gradual acidification of the supernatant induces liquid-liquid phase separation resulting in spherical protein-rich droplets suspended in protein poor continuous phase.

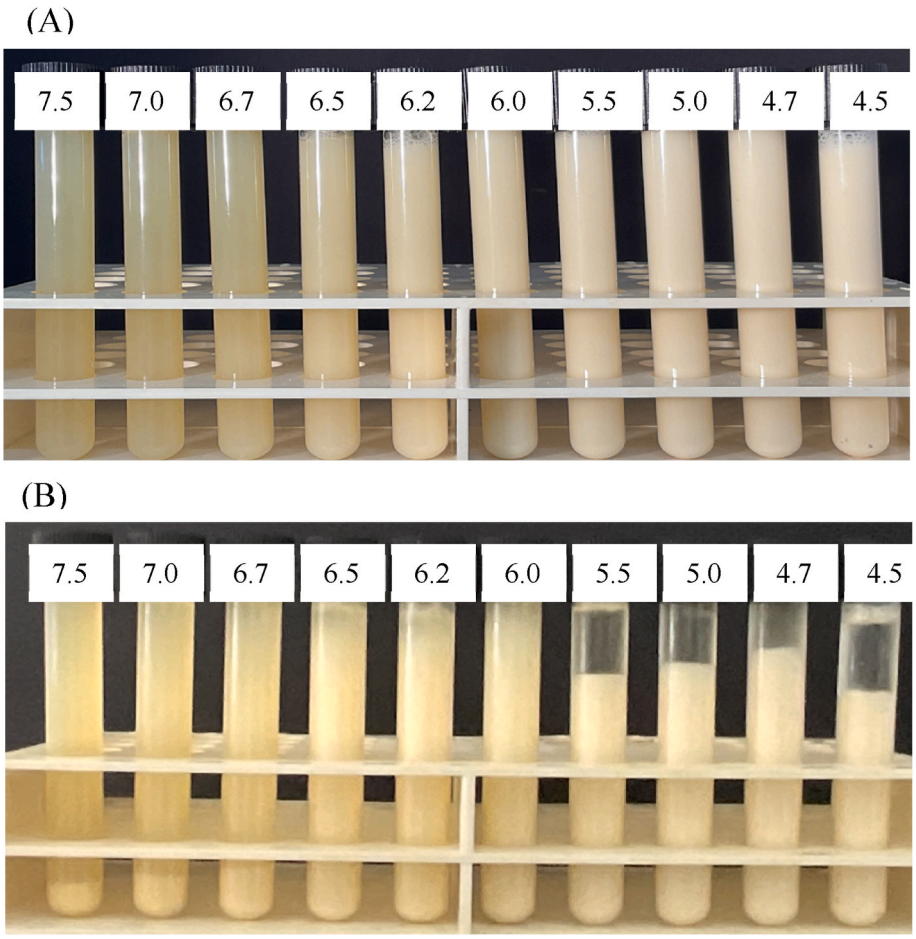

Fig. 2. Macroscopic images of yellow pea protein dispersions at various pH values, as indicated. (top) immediately after preparation (bottom) After overnight storage at 4 °C.

2.5. Dry matter mass balance analysis

After centrifugation of the flour dispersion, the soluble fraction of protein dispersion in alkali conditions was analysed as total (initial) dry matter M_T. Acidifying this alkali dispersion led to forming an insoluble fraction of proteins in the form of protein clusters and/or colloidal-sized particles. The insoluble fraction was centrifuged (17000 RCF, 30 min, 294K) to separate the protein soluble phase as supernatant and insoluble particles and clusters as pellets. The supernatant, containing soluble fractions, was analysed. This soluble mass is referred to as M_S, and the mass of the pellet containing the insoluble protein fraction (particles and particle clusters) was represented as M_P. A measure for the mass ratio of relative dry matter in %w/w, MR, of the mass of the supernatant versus that of the total (initial) dry matter content is given in eq. (1). All measurements were performed in triplicate.

$$M_R = \frac{M_P}{M_T} \times 100\% \quad (1)$$

The experiments used defatted soy flour. However, the yellow pea and fava bean flours were not defatted. Their fat content is reported to be around 1–3 %w/w fat (Krul, 2019; Mayer Labba et al., 2021; Kornet et al., 2020).

The protein content in legume flours was determined by analysing the nitrogen content. (Flash EA 1112 series DUMAS (Interscience, Breda, The Netherlands)). A constant value of 5.7 was used as a nitrogen-to-protein conversion factor for all proteins, i.e., from soy, yellow peas, and fava beans. The protein composition in flour alters according to climate and light intensity conditions during growth and soil characteristics. Hence, a range of nitrogen-to-protein conversion values can be found in literature ranging from 5.1 to 6.25 for different legume flours (Boisen et al., 1987; De Almeida Costa et al., 2006; Mossé, 1990; Pelgrom et al., 2013). All nitrogen content measurements were done in triplicates to determine protein content.

2.6. SDS - PAGE

The soluble protein dispersions were prepared by centrifuging alkali flour dispersions (17000 RCF, 30 min, 294 K). pH 6.0 particles containing protein dispersions were prepared according to the sample preparation method discussed in section 2.2 (Sample preparation). The pellet was composed of particles or particle clusters. Subsequently, a phosphate buffer (pH 8.0, 100 mM) was used to solubilise the pellet containing coacervates overnight at 4 °C. The final protein concentration of the samples was adjusted between 0.08 and 0.03 %w/w by diluting using a phosphate buffer (pH 8.0, 100 mM).

Reducing SDS-PAGE was conducted using a 4–12 % BisTris gel and a 20x diluted MES SDS running buffer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States). A 15 μL LDS sample running buffer was added to a 45 μL sample for non-reducing conditions. Six μL of 500 mM dithiothreitol (DTT) was added to the 39 μL sample and 15 μL LDS sample running buffer to conduct reducing conditions. The sample mixtures were heated to 70 °C for 10 min, and then 15 μL of the sample supernatants were loaded into each well. In one of the wells, ten μL of marker Thermo Mark12 Unstained standard marker (2.5–200 kDa) was loaded. Electrophoresis was performed in an XCell Surelock Mini-Cell (Thermo Fisher Scientific Inc., United States) for 35 min at a constant

voltage difference (200 V). The gels were subsequently stained using SimplyBlue SafeStain and washed with a 20 %w/w NaCl solution. These gels were scanned the next day with a Biorad Gel Doc EZ Imager (Bio-Rad, United States).

3. Results and discussion

3.1. Process of protein coacervation from legume flour

A schematic representation of the procedure for inducing liquid-liquid phase separation (simple coacervation) starting from legume flour is shown in Fig. 1. Legume flour was initially dispersed in slightly alkaline conditions (pH 8 to 8.3), effectively solubilising the proteins (Fig. 1a). Unless mentioned otherwise, this is referred to as flour dispersion in the following text. The alkaline flour dispersion was centrifuged to remove insoluble fractions such as fibres, starch granules, and insoluble proteins (Fig. 1b), resulting in a clear supernatant called protein dispersion. Finally, the protein-rich supernatant was decanted and slowly stepwise acidified using 1M H_2SO_4 to a range of more acidic pH values (pH 7.0 – pH 4.5) (Fig. 1c). This acidification induced the formation of protein-rich particles (or droplets), often called coacervates, dispersed in a protein-poor continuous phase.

The first part of the results pertains to pea protein, followed by soy and fava proteins. Some results are not discussed in the main text but are available in the supplementary information.

3.2. Macroscopic observations

The macroscopic appearance of the various protein dispersions in the case of yellow pea flour is illustrated in Fig. 2. The figure shows the appearance of the dispersions for pH values from 7.5 to 4.5, both shortly after acidification and after overnight storage. As the pH was decreased towards the isoelectric point, we observed an increase in turbidity, as seen in Fig. 2A. Initially, translucent (pH 7.5) samples were transformed into milky white samples at lower pH levels (pH 4.5). Fig. 2B shows that sedimentation occurs in samples stored overnight for the samples at pH values below 5.5.

We find a similar pattern for the macroscopic appearance of soy and fava bean flour dispersions after acidification, before and after storage (see figure supplementary information (SI)-1 and SI-2). To better understand the changes in the structure of the dispersions after the slow and stepwise acidification, we next turn to microscopic analysis.

3.3. Optical microscopy

Fig. 3 shows representative micrographs demonstrating the structural evolution in yellow pea protein dispersions upon stepwise acidification (pH 7.3, 6.5, 5.0). We use brightfield transmission optical microscopy and Confocal Scanning Laser Microscopy (CSLM), using Rhodamine B as a non-covalent fluorescent stain for the proteins. Three different pH regimes can be distinguished. The representative micrographs illustrate them for the case of yellow pea flour shown in Fig. 3. At high pH, neutral to slightly basic, the proteins are soluble, as illustrated by the micrographs for the pH 7.3 sample; for a small range of intermediate pH values, the proteins form spherical particles (presumably liquid droplets), as illustrated by the micrograph for the pH 6.5 sample. We tentatively identify this regime as the simple coacervation regime. Finally, the (initially) spherical particles form large clusters at low pH, as illustrated by the micrograph for the pH 5.0 sample. We refer to this last regime as the cluster regime.

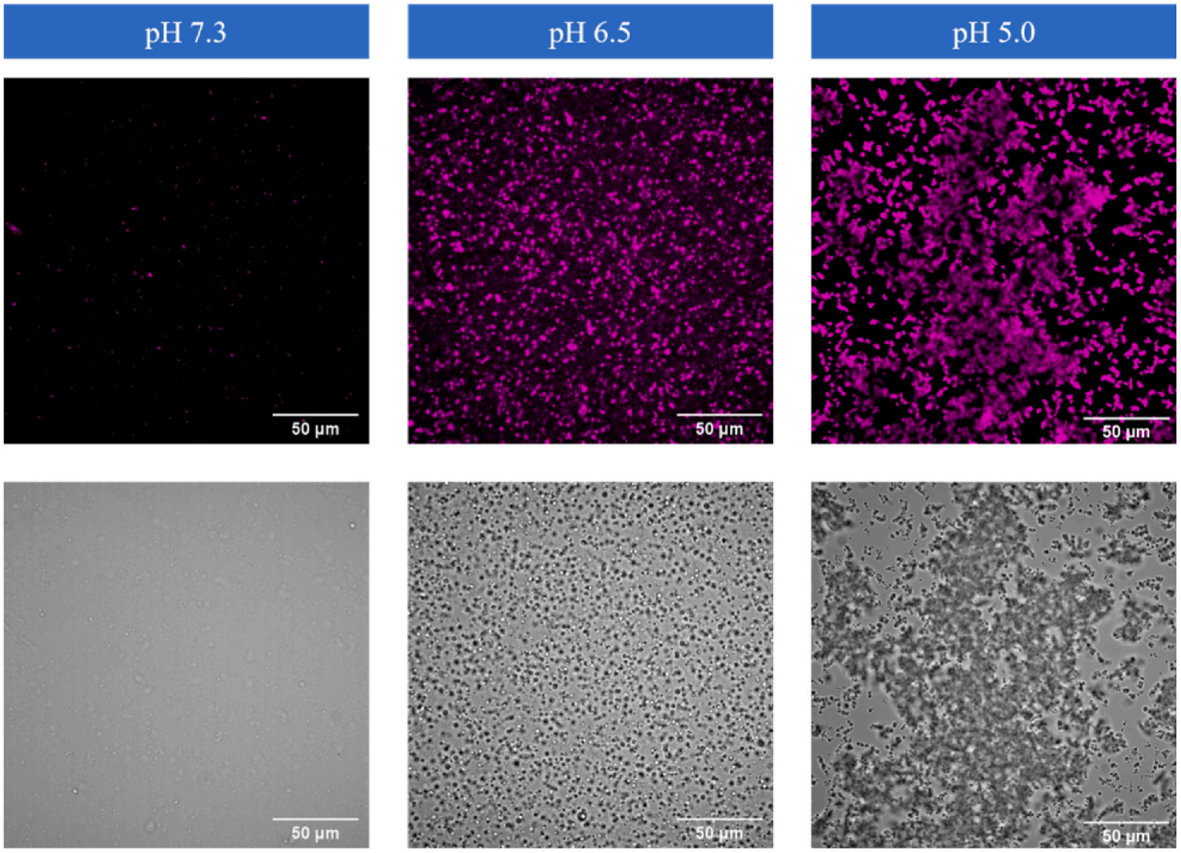

Fig. 3. Micrographs of protein dispersions at different pH values prepared from 15%w/w yellow pea flour dispersion. Both Confocal Laser Scanning Microscopy (CLSM) and transmission channels were used. For CLSM, proteins were non-covalently stained using Rhodamine B (Magenta). Top row: CLSM micrographs at pH 7.3, 6.5 and 5.0. Bottom row: bright field micrographs. Scale bar: 50 µm.

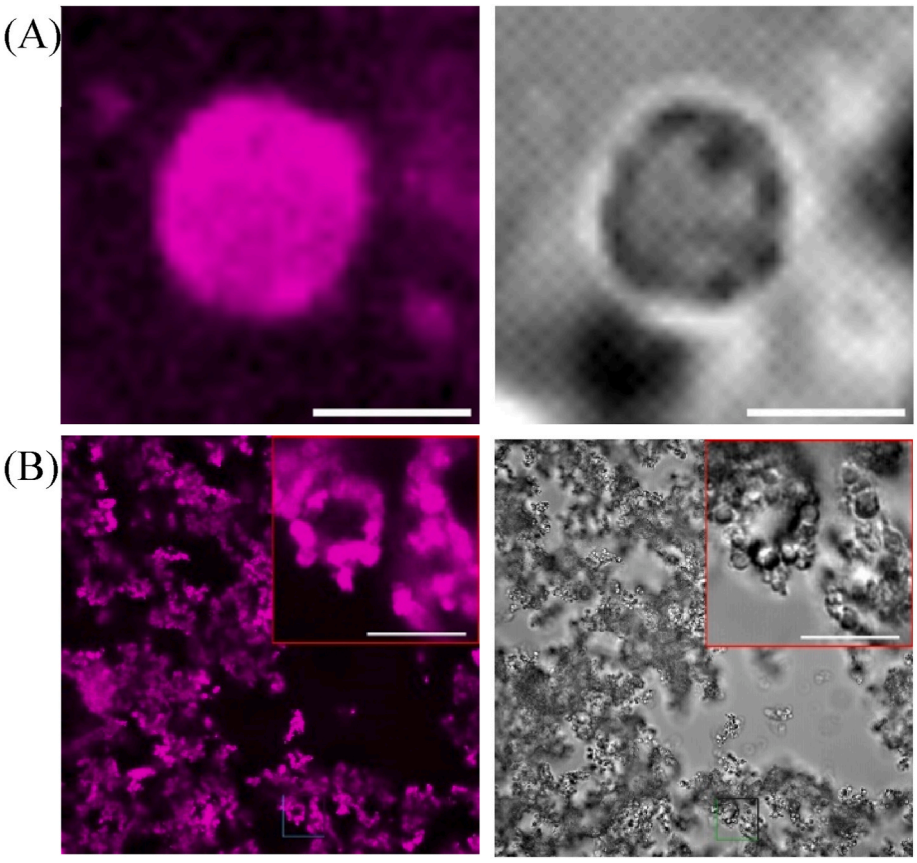

Fig. 4. More detailed (left: CLSM, right: brightfield transmission microscopy) images of structures found during acidification of yellow pea protein dispersions prepared from 15%w/w flour dispersion. (A) Roughly spherical protein particles at pH 6.5. Scale bar 2 μm. (B) Clusters of protein particles at pH 4.8. Scale bar 50 μm.

From a much larger set of micrographs, for the case of yellow pea flour (Figs. SI–3), we could estimate the phase transition boundaries for the three regimes: the soluble regime emerges for pH > 6.7, followed by droplet formation in the intermediate ranges of pH 6.7–5.8 and droplet clustering from pH 5.8–4.8. Similarly, from sets of micrographs for the cases of soy flour (Figs. SI–2) and fava bean flour (Figs. SI–4), we could estimate that a similar soluble phase corresponds to pH > 6.7, the simple coacervate regime occurs for pH 6.7–5.8, followed by the cluster phase transition pH boundaries for these cases as well.

The particles under alkali and neutral conditions (pH 7.0 – Figs. SI–3) are irregular clusters of submicron sizes and, hence, are not always clearly visible using optical microscopy techniques. Thus, the first boundary depicting the onset of the formation of coacervate droplets can only be approximately delineated using microscopy measurements. This should be further confirmed more accurately using particle size distribution measurements. On the other hand, the second boundary, depicting the transition from the soluble phase to an onset of clusters of coacervate droplets, can best be determined from undiluted samples using microscopy. This is because the clusters might fall apart due to the dilution in particle size measurements.

To better illustrate the nature of the particles in the simple coacervate regime, we refer to the higher-resolution micrograph of a pea protein particle at pH 6.5 in Fig. 4 (A). The micrograph clearly illustrates the spherical nature of the protein particles and the (from this scale onwards) homogeneous protein distribution within the spherical particle. Fig. 4 (B) shows a more detailed micrograph of yellow pea protein particles at a lower pH value of pH 4.8, which have undergone aggregation. The micrograph shows that the protein particles have clustered into an irregular structure. The inset highlights that (at least in the case of our slow acidification process) the clusters seem to consist of nearly spherical protein particles. This suggests that during slow acidification, (presumably liquid) spherical protein particles first form and later (at lower pH values) form clusters.

3.4. Change of particle size upon acidification

We use particle size determination based on laser light diffraction for a more quantitative analysis of particle size distribution. Fig. 5 shows how the average particle diameter changes upon acidification for different initial flour concentrations. Fig. 5A, B, and C present sizes versus pH for the case of soy, yellow pea, and fava bean. For each case, data is included for initial flour concentrations of 15 %w/w, 18 %w/w, and 21 %w/w. It should be noted that samples are highly diluted and mechanically agitated in the particle sizing equipment. This could cause large aggregates, such as those observed at low pH in microscopy, to break up. It is clear from Fig. 5 that, for all protein sources, the particle diameter increases monotonically upon slow acidification from pH 7.0 to 4.8. The three pH regimes distinguished in microscopy can likewise be observed in the particle sizing. Table 1 gives the pH boundaries for the soluble, coacervate, and cluster regimes (more detailed data can be found in SI-2 – SI-7). These correspond well to those derived from optical microscopy and clarify the boundary between submicron and observable protein particles.

We also find a clear pattern in the evolution of the particle size distribution curves upon acidification. Individual particle size distribution curves for various pH values for soy, yellow pea, and fava bean are

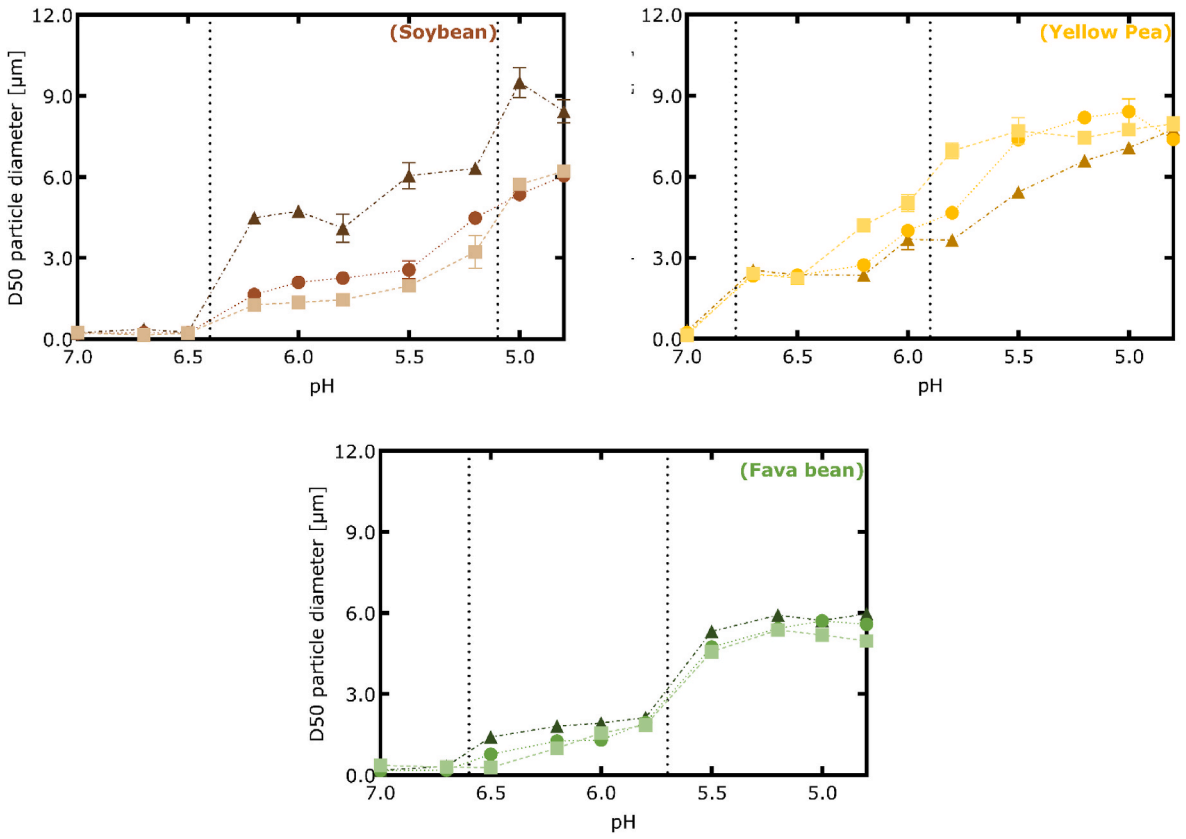

Fig. 5. Volume-based particle sizes (D50) as a function of pH during slow stepwise acidification of different legume protein dispersions at flour dispersion concentrations of 15 %w/w (■), 18 %w/w (●) and 21 %w/w (▲) dry matter. Protein sources: (A) soybean, (B) yellow pea, (C) fava bean. The dotted line represents the phase transition from (left · line) sub-micron sizes to protein particle phase and (right · line) from the particle phase to particle clustering phase.

Table 1
The phase transition pH boundaries for different protein sources (soybean, yellow pea, and fava bean proteins). The protein dispersions, upon acidification from pH 7.0–4.8, show the formation of 3 distinct phases: (i) soluble phase, (ii) formation of protein particles, (iii) clustering of protein particles in the case of all protein sources at different pH values.

Protein source	Soluble (sub-micron)	Individual Protein particles	Clustered protein particles
Soybean proteins	pH 7.0 – pH 6.5	pH 6.2 – pH 5.5	pH 5.2 – pH 4.8
Yellow pea proteins	pH 7.0	pH 6.7 – pH 5.8	pH 5.5 – pH 4.8
Fava bean proteins	pH 7.0 – pH 6.7	pH 6.5 – pH 5.8	pH 5.5 – pH 4.8

shown in Figs. SI–5, SI-6, and SI-7. Monomodal peaks predominate for the solution and coacervation regimes, while multimodal peaks are observed in the clustering regime. This phenomenon can be attributed, in part, to the agitation mechanism and dilution effect inherent in the equipment used, wherein the shear forces generated by stirring serve to disrupt the large clusters that we observed using microscopy.

The diameters of the spherical protein particles in the coacervation pH range are source dependent. We find diameters in the range of

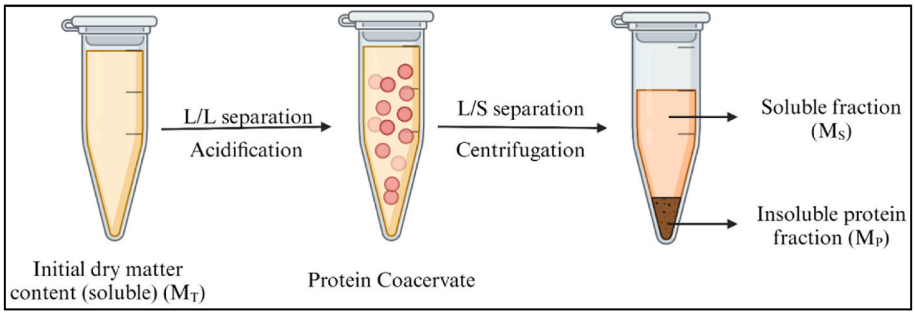

Fig. 6. Schematic illustrating the method for analysing the mass balance. The process starts with measuring the protein dispersion's total (initial) dry matter (M_T), followed by acidification to form protein particles or clusters. It concludes with centrifugation to separate the mixture into a pellet containing the protein-rich particles or clusters fraction (dry matter content represented as M_P) and a protein-poor supernatant phase (dry matter weight M_S). Equations (1) and (2) are used to calculate the insoluble dry matter content (M_P).

Table 2
Soluble protein content (%w/w in protein dispersions) after centrifugation of alkaline flour dispersions prepared at concentrations of 15–21 g/100g flour of soybean, yellow pea, and fava beans.

Protein content after various steps	Soybeans	Yellow Pea	Fava beans
Flour	50.56 ± 0.59	18.7 ± 0.30	27.29 ± 0.24
15% w/w stock	7.85 ± 0.08	2.97 ± 0.01	4.39 ± 0.01
18% w/w stock	9.56 ± 0.07	4.51 ± 0.05	5.55 ± 0.08
21% w/w stock	10.9 ± 0.06	4.71 ± 0.01	6.32 ± 0.10
pH 4.5 continuous phase	1.89 ± 0.02	0.94 ± 0.01	1.02 ± 0.01

1.7–4.7 μm for soy. Yellow pea proteins form particles of 2–5 μm, and fava bean proteins form minuscule particles of 1–2 μm.

The pH boundaries obtained from microscopic and particle size distribution analysis for the three flours considered here are given in Table 1. The first boundary (soluble or sub-micron clusters to protein particles) could be delineated using the particle size measurements. In contrast, the second boundary (protein particle to particle clusters) could be delineated from the microscopy results. Our findings indicate that these pH boundaries shift away from neutral conditions towards the isoelectric point as protein concentration increases. Defatted soy flour contains 50.56 %w/w protein, fava flour contains 27.29 %w/w, and pea contains 18.70 %w/w protein. This leads to different protein concentrations for the same flour concentrations in protein dispersions. Our findings indicate that yellow pea protein particles were formed in slightly acidic conditions (pH 6.7). In contrast, fava bean protein particles were formed in more acidic conditions (pH 6.5), and soy protein particles were formed at an even lower pH (pH 6.2). A similar trend is observed in the clustering of protein particles: soy dispersions seem to form clusters starting at pH 5.0, whereas fava beans and yellow peas form clusters starting at pH 5.5. We employed laser diffraction to analyse particle size distribution and investigate these results further, particularly to delineate the transition from the sub-micron-sized cluster phase to protein particle formation. The results for the pH transition range are shown in Table 1. A more detailed data can be found in SI-5 – SI-7.

3.5. Mass balance and protein solubility upon acidification

First, we obtain a mass balance for both the dry matter and, more specifically, for proteins. Our process starts by solubilising the different legume proteins in alkaline pH and making protein dispersions (Fig. 1). The total solids present as soluble fractions in the protein dispersion (supernatant after centrifuging flour dispersions) is called the initial dry matter (M_T). After the slow and stepwise acidification, for pH values in the coacervate and cluster regions, the dry matter can be split into two parts: "P", which can be spun down by centrifugation, and the supernatant part "S", which cannot be spun down. Hence, the total mass (M_T) equals $M_P + M_S$ (See Fig. 6). As a function of pH, results for the relative amount of dry matter M_R present in the coacervate particles and cluster were calculated using equation (1) of Section 2.5 analysis. Table 2 presents the percentage of soluble proteins by weight in protein dispersions, following the centrifugation of alkaline flour dispersions prepared with soybean, yellow pea, and fava beans at concentrations ranging from 15 to 21 g per 100 g of flour. Fig. 7 shows the dry matter results for various sources. The relative dry matter content in particles monotonically increases as the pH decreases. Next, we consider the possibility of partitioning proteins of different types present in the flours

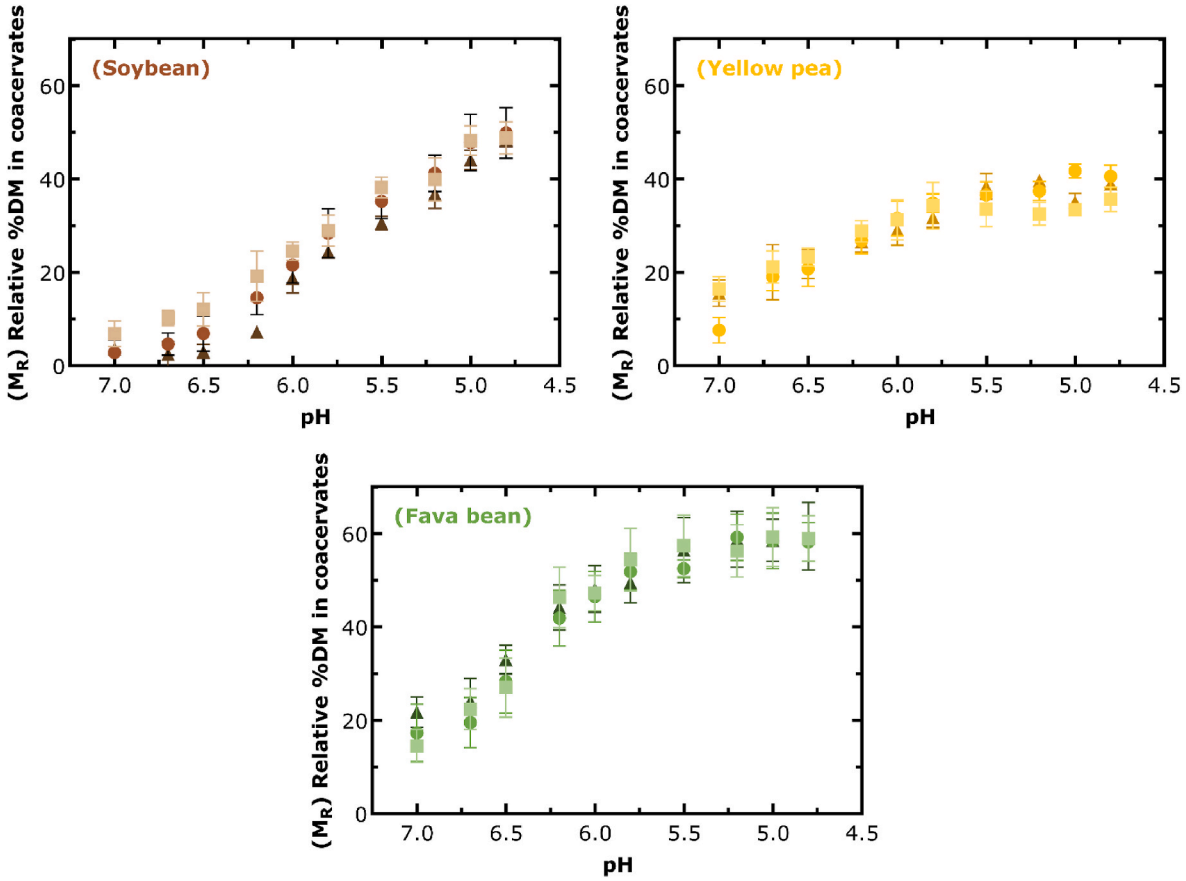

Fig. 7. Relative dry matter content M_R of protein-rich particle phase, $M_R = (M_P/M_T) \times 100$, where M_T is total (initial) dry matter of the protein dispersion ($M_T = M_P + M_S$), M_P dry mass of dispersed particle phase, M_S is dry mass of continuous phase. Initial concentration of flour in dispersions: 15 %w/w (■), 18 %w/w (●) and 21 %w/w (▲). Protein sources: (A) Soybean, (B) Yellow pea, (C) Fava beans.

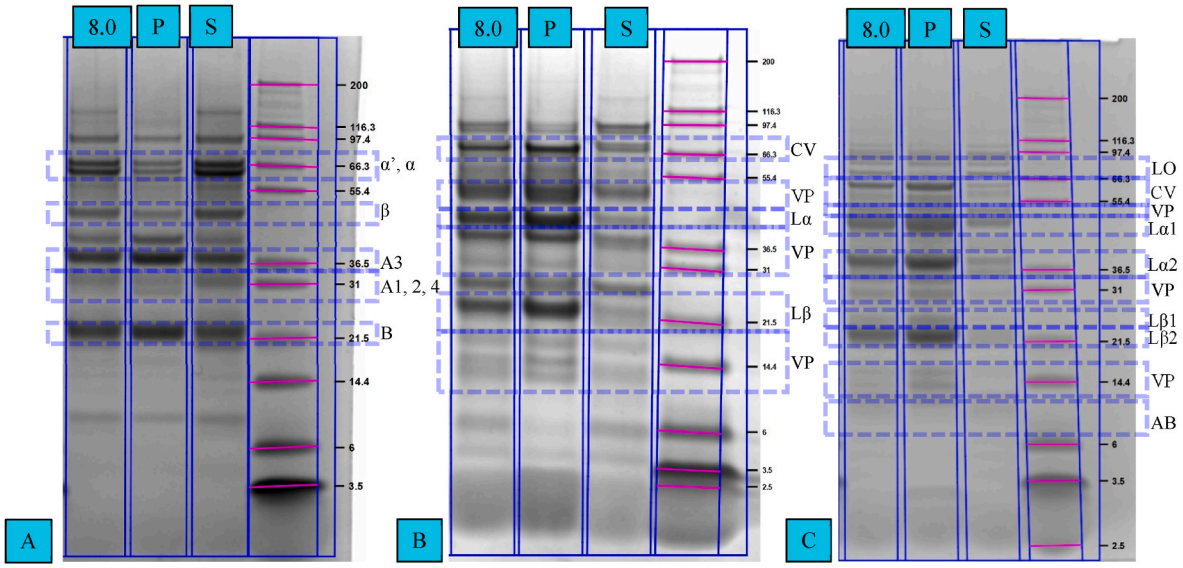

Fig. 8. SDS-PAGE electrophoresis analysis (reducing conditions) of partitioning of proteins acidified to pH 6.0 (within the particle forming range) over continuous phase forming the supernatant phases ("S") and centrifuged dispersed particle phase ("P") that was resolubilized at pH 8.0. Protein sources: (A) Soybean protein (β-CG: Conglycinin subunit, G: Glycinin subunit, α′ and α: Conglycinin subunits, β: Conglycinin subunit, A1 – A4: Acidic subunit (Glycinin), B: Basic subunits (Glycinin)), (B) Yellow pea protein (CV: convicilin subunit, VP: vicilin polypeptides, Lα: acidic legumin, polypeptides, L β: basic legumin polypeptides) (C) Fava bean protein. (LO: Lipoxygenases, CV: Convicilin, VP: Vicilin Polypeptides, Lα1 and Lα2: acidic legumin polypeptides, L β 1 and Lβ 2: basic legumin polypeptides).

over the particle ("P") and supernatant ("S") phases.

3.6. SDS-PAGE analysis of soy, yellow pea, and fava proteins under reducing and non-reducing conditions

Fig. 8A, B, and C present SDS-PAGE analyses under reducing conditions of soy, yellow pea, and fava bean proteins in the particle ("P") and supernatant ("S") phases. Proteins extracted from legume flours were initially solubilised to make protein dispersion at an alkaline pH of 8. Subsequently, the pH slowly decreased to pH 6.0, and the particle ("P") and supernatant ("S") samples were obtained by centrifugation. Before electrophoresis, these fractions were solubilised in a pH 8 buffer. A protein standard marker, with molecular weights ranging from 2.5 to 200 kDa, is shown in the last lanes of the gels. The protein fractions were identified according to the previous study on reducing conditions of these legumes (Kornet et al., 2020; Xia et al., 2021; Shrestha et al., 2023; Berrazaga et al., 2019; Warsame et al., 2020).

In Fig. 8A, reducing SDS-electrophoresis for the case of soy indicates the presence of β-conglycinin in all sample lanes. While the lane intensities for the pH 8 and the supernatant (continuous phase) at pH 6 ("S") are comparable, the intensity of these bands for the dispersed phase particles at pH 6 ("P") fraction is lower. In contrast, lanes for the glycinin acidic subunits A3 are most pronounced for the particle phase ("P"), which makes it seem that glycinin is enriched in the particle phase. In contrast, β-conglycinin is enriched in the continuous phase. SDS-PAGE analysis (reducing conditions) for yellow peas is shown in Fig. 8B. For this case, we find that the α and β subunits of the Legumin proteins are enriched in the particle phase ("P"), whereas vicilin is enriched in the supernatant phase ("S"). Convicilin does not seem to show robust partitioning. Finally, SDS-PAGE analysis (reducing conditions) for the case of fava bean is shown in Fig. 8C. Here, the legumin subunits and the convicilin appear to partition strongly to the particle phase ("P"). SDS-PAGE in non-reducing conditions also confer these results (Figs. SI–8).

In a comparable investigation, similar pH ranges conducive to the formation of various particle morphologies using defatted soy flour: submicron particles (pH 6.8–6.18), spherical protein particles (pH 5.85–6.15), and particle clusters (pH < 5.6) (Lui et al., 2007). The study also detailed the composition of the droplets formed at pH 6.0; similar to our current results, these droplets predominantly consisted of the glycinin fraction (approximately 85%), with the remainder comprising the beta-conglycinin fraction (approximately 15%). At pH 6.4, droplets are predominantly composed of the glycinin fraction. However, as the pH nears the isoelectric point, the compositional dynamics shift, resulting in a decrease in glycinin purity and a corresponding increase in the beta-conglycinin fraction. The isoelectric point of soy glycinin is 4.65–4.75 (Chen et al., 2017; Koshiyama, 1972) and that of the Beta conglycinin subunit is reported as: α – 4.90; α′ – 5.18; β – 5.66–6.00 (Huu Thanh & Shibasaki, 1977).

In a study on pure glycinin protein fraction at low ionic strength, increasing turbidity is observed as the pH decreases from pH 7.4–6.3, indicating the onset of protein insolubilisation. A marked reduction in solubility is observed below pH 6.8, with significant and visible aggregate formation occurring when the pH drops below 6.4. These results are similar to our study on unpurified soy protein mixtures. Notably, these aggregates are capable of re-solubilising when the pH is increased. Furthermore, the study shows that increasing the content of β-conglycinin reduces the rate of phase separation and droplet formation during this process (Chen et al., 2017). Our findings, in conjunction with previous studies, suggest that glycinin primarily drives the liquid-liquid phase separation process. However, the resulting droplets are not exclusively composed of glycinin, indicating the involvement of other protein components, such as beta-conglycinin, in forming these phases.

In Cochereau's research, an analogous trend is observed to that in the current study, where the phase transition boundary for the formation of spherical protein-rich domains in samples containing 2–4% protein exhibits a shift from neutral pH toward the isoelectric point (Cochereau et al., 2019). Notably, this trend persists even with a reduction in concentration, suggesting the existence of a critical threshold. Above and below this threshold, the phase transition boundary for particle formation diminishes.

4. Concluding remarks

Salt addition and acidification can induce liquid-liquid phase separation since they both affect electrostatic interactions. Given the current focus on minimising salt content in foods, this study focuses on acid-induced phase separation. Commercial legume protein concentrates

and isolates there are often associated with a relatively high level of denaturation (Doshi et al., 2024). Denatured legume proteins are expected to be limited in their susceptibility to acid-induced phase separation. Therefore, we utilise flour dispersions in which proteins are minimally processed, preserving their native conformation and solubility.

Simple coacervation in legume proteins, obtained by alkaline extraction, shows the generality of this liquid-liquid phase separation phenomenon for legume storage proteins, which we have alluded to before (Doshi et al., 2024). While existing literature (Chen et al., 2016b, 2017, 2018, 2020a, 2020b; Jiang et al., 2010; Lazko et al., 2004; Lui et al., 2007; Yagasaki et al., 1997) primarily documents this behaviour in purified protein and protein mixtures, like soy glycinin and pea protein isolate (Chen, Nicolai, et al., 2020; Cochereau et al., 2019), our findings extend these observations to mixtures of protein fractions freshly extracted in alkaline conditions from whole legume flours. Importantly, our results demonstrate that phase separation can be achieved by adding salt, as commonly reported, and by simply altering the sample's pH as a general phenomenon in legume-based protein mixtures. Interestingly, the observed non-coalescence of droplets at acidic pH suggests potential stabilisation at the interface, likely due to the multicomponent nature of the system. Interestingly, the resistance against coalescence of coacervates at acidic pH strongly suggests the presence of proteins (or a mixture of different proteins) at the interface. Initial observations indicated that these coacervates do not coalesce at low centrifugal forces (800 RCF, 10 min, 294 K) and can be redispersed after centrifugation, further supporting the presence of stabilising compounds at the coacervates' surface.

We also showed partitioning effects over the (coacervate) particle and supernatant phases (Chen et al., 2017; Yagasaki et al., 1997). It will take much more elaborate work to come to a quantitative description of what determines the partitioning.

We expect our study to benefit further work on exploiting protein liquid-liquid phase separation in foods, following the directions pointed out in Tombs' early work (Tombs et al., 1974). Our observations may form a basis for creating protein ingredients such as hollow microcapsules, microgel particles and other structures via heat-set gelation directly from legume flours, concentrates and isolates.

CRediT authorship contribution statement

Nirzar Doshi: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Paul Venema:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Erik van der Linden:** Writing – review & editing, Supervision, Funding acquisition. **Renko de Vries:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used OpenAI – ChatGPT in order to manuscript's readability and language. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work is part of the project 'Clean label solutions for structuring plant-based foods' co-financed by the Top Consortium for Knowledge and Innovation Agri & Food by the Dutch Ministry of Economic Affairs under contract number LWV20.68.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.110927.

Data availability

Data will be made available on request.

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