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Structural evolution of pea-derived albumins during pH and heat treatment studied with light and X-ray scattering

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ABSTRACT

Pea albumins are found in the side stream during the isolation of pea proteins. They are soluble at acidic pH and have functional properties which differ from their globulin counterparts. In this study, we have investigated the aggregation and structural changes occurring to pea albumins under different environmental conditions, using a combination of size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALS) and small-angle X-ray scattering (SAXS). Albumins were extracted from a dry fractionated pea protein concentrate by precipitating the globulin fraction at acidic pH. The albumins were then studied at different pH (3, 4, 4.5, 7, 7.5, and 8) values. The effect of heating at 90 °C for 1, 3, and 5 min on their structural changes was investigated using SAXS. In addition, size exclusion of the albumins showed 4 distinct populations, depending on pH and heating conditions, with two large aggregates peaks (~250 kDa): a dimer peak (~24 kDa) containing predominantly pea albumin 2 (PA2), and a monomer peak of a molar mass of about 12 kDa (PA1). X-ray scattering intensities as a function of q were modeled as polydisperse spheres, and their aggregation was followed as a function of heating time. Albumins was most stable at pH 3, showing no aggregation during heat treatment. While albumins at pH 7.5 and 8 showed aggregation after heating, solutions at pH 4, 4.5, and 7 already contained aggregates even before heating. This work provides new knowledge on the overall structural development of albumins under different environmental conditions, improving our ability to employ these as future ingredients in foods.

1. Introduction

Albumins are small, globular and soluble proteins. Those derived from plant sources, are currently attracting significant attention, despite being present in a smaller concentration compared to the other storage proteins, the globulins (Kim, Wang, & Selomulya, 2020). Plant-based albumins are soluble at acidic pH, therefore they are usually found in high proportion in the side streams during the isoelectric precipitation of plant protein isolates. Understanding their properties will increase the value of a processing waste stream and contribute to more circular processes. Although research reports on plant-derived albumins are limited, few preliminary studies pointed to their potential to be used as technologically functional proteins in foods (Cheung, Wanasundara, & Nickerson, 2015; Wong, Pitts, Jayasena, & Johnson, 2013; Yang & Sagis, 2021). For example, the albumins from pea possess emulsifying and foaming properties (Lu, Quillien, & Popineau, 2000; Yang, de Wit, et al., 2022; Yang, Kornet, et al., 2022), as well as gel-forming properties (Kornet et al., 2021).

Amongst the storage proteins in pea, globulins account for approximately 60–80 % of the total protein. The side stream resulting from the precipitation of the globulins does not only contain soluble albumin fractions, but also other molecules, such as soluble carbohydrates and phenolic components, which can negatively affect protein digestibility or sensory attributes such as color and flavor (Dhaliwal, Salaria, & Kaushik, 2021). These unwanted molecules and active enzymes may be reduced, at least in part, by additional processing steps, e.g., filtration (Lu et al., 2000), or inactivated by other processes, like heating and fermentation (Samtiya, Aluko, & Dhewa, 2020).

Globular proteins' structure can be significantly affected by pH, ionic strength, or heating. These changes are critical to effectively evaluate how to best utilize these proteins as functional food ingredients. Scattering experiments are often employed to gather information about protein structural rearrangements at the molecular as well as at the supramolecular scale. Small-angle X-ray scattering (SAXS) has been

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widely employed in the study of structural properties and characterization of complex protein structures at multiple length scales, for example, in milk protein (Li et al., 2021, 2022; Pedersen, Moller, Raak, & Corredig, 2022), and plant proteins (Chen, Kuzmenko, Ilavsky, Pinho, & Campanella, 2022; Khaliq et al., 2017; Miles, Morris, Carroll, Wright, & Newby, 1985). By providing insights into the arrangement of molecules and their interactions at scales ranging from ca. 1 to 200 nm, SAXS can probe the structural evolution of protein solutions without disruption, in their natural aqueous environment, further providing a unique perspective on the structural organization of the proteins. In addition, a comprehensive understanding of the aggregation dynamics *in situ* can be obtained when combining SAXS with other complementary techniques, such as light scattering or size exclusion chromatography.

To our knowledge, no systematic study has been reported on how pea-derived albumins are affected by the changes of pH and heat treatment; and this is crucial for understanding their properties and potential applications. In the present study, the fine details of pea albumins heat-induced structural evolution were investigated in a wide range of pH (3–8). These new insights will improve our ability to utilize pea albumins as ingredients in new food formulations.

2. Material and methods

2.1. Materials

Pea protein concentrate (55 % protein content, w/w) was kindly provided by Vestkorn A/S (Holstebro, Denmark). The concentrate was processed through milling and air classification. Hydrochloric acid and sodium hydroxide are analytical grades and obtained from Merck (Merck Life Science A/S, Søborg, Denmark).

2.2. Sample preparation

The separation of albumin from globulins was carried out as previously reported in the literature (Sari, Mulder, Sanders, & Bruins, 2015), with some minor changes, namely, the use of pea protein concentrate instead of whole pea flour. In brief, pea protein concentrate was first dispersed in MilliQ water (1:10, w:v) and adjusted to pH 9 using 1 M NaOH, and then solubilized for at least 2 h using a magnetic stirrer (600 rpm). The extract was then centrifuged at 4500× g, 30 min, 20 °C to remove the insoluble components. The supernatant was collected and adjusted to pH 4.5 using 1 M HCl at room temperature, to separate the albumin fraction from the globulins, and kept stirring (400 rpm) for 2 h. Subsequently, a second centrifugation was conducted with 10,000× g for 30 min at 4 °C. The supernatant was then dialyzed using 6–8 kDa membrane for 3 days, including changing water 3 times each day. The final pH of the supernatant was around 4.8. Finally, the supernatant after dialysis was freeze-dried, labeled as albumins and stored at −20 °C until further use. The powder contained 70 % (w/w) protein, as measured using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, U.S.A.), the rest of around 30 % were carbohydrates& polyphenols as measured using Phenol-Sulfuric Acid Method (Nielsen, 2010).

Freeze-dried samples were suspended in MilliQ water at 10 mg protein/mL and stirred (400 rpm) for at least 2 h at room temperature. The solutions were then adjusted to different pH values (3, 4, 4.5, 7, 7.5, and 8) with 1 M HCl and NaOH respectively. Then each solution was heated for 1, 3, and 5 min at 90 °C.

2.3. Zeta potential

The zeta potential of the various solutions were measured by dynamic light scattering (Malvern Zetasizer Nano, Malvern instruments Ltd, Malvern,UK). Samples were diluted (approx 10 times) in 50 mM NaCl and filtered through a 0.45 μm Millex-GV filter unit (Merck Millipore Ltd., Cork, Ireland). Each sample was run in triplicate (each run was an average of 100 measurements) at 20 °C.

2.4. Size-exclusion chromatography coupled with a multi-angle laser light scattering (SEC-MALS)

The composition and molecular mass of the albumin fractions were measured using SEC-MALS. Briefly, protein solutions, heated or unheated (10 mg protein/mL) were filtered through 0.45 μm Whatman filters (PVDF) and then 30 μL were injected in the HPSEC system equipped with a Guard Cartridge (GFC 4000, 4 x 3.0 mm ID) and a Yarra SEC-4000 column (300 x 7.8 mm, 3 μm) (Phenomenex, Værløse, Danmark) connected in series and set at 25 °C. The mobile phase used was 0.02 μm filtered 50 mM sodium phosphate buffer (38 mM Na_2HPO_4 and 12 mM NaH_2PO_4) containing 150 mM NaCl and 0.02 % sodium azide (pH 7.2) at a flow rate of 0.5 mL/min. Data analysis was conducted with the ASTRA software (version 8.1; Wyatt Technology, Santa Barbara, CA), and the weight average molecular mass (Mw) was derived using a second-order Berry fit.

2.5. Small-angle X-ray scattering (SAXS)

Small-angle X-ray scattering (SAXS) was used to measure the structural changes of pea albumins subject to different heating and pH using a laboratory setup (Nano-inXider, Xenocs SAS, Grenoble, France), equipped with a Rigaku (Rigaku-Denki, Co., Tokyo, Japan) 40 W microfocused Cu K_α source with a 1.54 Å wavelength and a Pilatus detector from Dectris (Baden, Switzerland). The resulting q-range was recorded from 0.004 to 0.3 $Å^{-1}$.

Samples were first loaded and sealed into borosilicate capillary tubes and followed by evacuation of 5 min in a desiccator at room temperature. Subsequently, the samples were measured at total acquisition time of 60 min at room temperature under the combined setting of very high resolution (beam size of 200 μm) for 15 min and medium resolution (beam size of 800 μm) for 45 min. The background buffer was obtained, for each sample through filtration using a 3 kDa membrane of centrifugal filter unit. Then data reduction was performed with the XSACT software for background subtraction.

The open source Monte Carlo regression package McSAS 1.3.1 was used to analyze the scattering curves and evaluate model fits (Breßler, Pauw, & Thünemann, 2015; Pauw, Pedersen, Tardif, Takata, & Iversen, 2013). McSAS uses a Monte Carlo based approach to fit the data using a polydisperse sphere distribution, but assuming no pre-determined shape of the distribution. It assumes a two-phase contrast system which we consider fulfilled here after background subtraction. The model was applied on the q range from 0.005 to 0.2 $Å^{-1}$.

2.6. Statistical analysis

The study used SPSS Statistics 28 (IBM, New York, USA) for statistical analysis. ANOVA (one-way analysis of variance) was done following Tukey's multiple comparison test based on a statistical significance level of $P < 0.05$.

3. Results and discussion

3.1. Zeta potential measurements

Fig. 1 summarizes the changes in the visual appearance of the pea albumin solution, as a function of pH, as well as their overall charge, reported as ξ-potential, of the proteins. The overall charge of the pea albumins was near neutral at pH 5. This pH agrees with the isoelectric point (pI) of pea albumins reported in the literature (Archut, Klost, Drusch, & Kastner, 2023). The overall protein charge was positive, reaching a maximum charge of +20 mV at acidic pH, and a maximum negative charge of −13 mV at pH above the isoelectric point. A better stability at high and low pH was expected because of the high

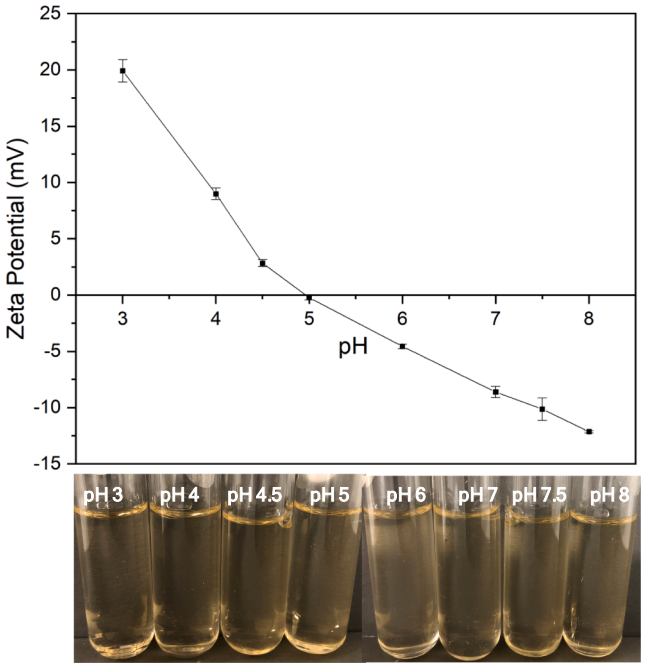

Fig. 1. Zeta potential and visual appearance of pea albumins solution (10 mg/ml) as a function of pH.

electrostatic repulsion between the proteins. For example, native β-Lactoglobulin (β-lg) exists as a mixture of monomers and dimers when far from its pI, while tends to form larger oligomeric structures, possibly octamers around its pI at low ionic strength (Verheul, Pedersen, Roefs, & de Kruif, 1999). As shown by the images in Fig. 1, the albumin solutions were clear regardless of pH.

3.2. Structural changes of albumins using SAXS and SEC-MALS during pH changes

All solutions were analyzed to evaluate the presence of soluble aggregates using SEC. Fig. 2 shows the elution based on light scattering (LS) and UV responses for the various albumin fractions adjusted to different pH values. Four main peaks were detected, and the molar mass (C) profile quantified by MALS corresponding to each of these peaks is also shown in Fig. 2. The molar mass of each peak was not different between samples, significantly affected by pH, but rather the distribution of the various peaks. At pH 3, the albumins are positively charged. They eluted in two major peaks (peak 3 and 4), corresponding to the two native albumin fractions: PA2 (reported in the literature to have a molecular mass ranging between 23 and 26 kDa) and PA1 in monomeric form, the latter reported with a much broader molecular mass of 4–18 kDa (Kornet et al., 2022; Lu et al., 2000; Yang, Kornet, et al., 2022). When measured by multi-angle laser light scattering, these peaks also showed a molecular mass within these reported ranges, namely, with peak 3 estimated at 25 KDa, and peak 4, PA1 at 12 KDa. These values did not change for the various pH treated samples.

Solutions prepared at pH 4 showed 2 additional peaks (peak 1 and 2), eluting earlier. This indicated the presence of a small population of soluble aggregates. The peak eluting at 21 min had a larger molecular mass, at about 47 kDa. This peak indicated that with a decrease in electrostatic repulsion, oligomers of the albumins appeared. It has indeed been reported that the subunits (PA2a and PA2b) of PA2 could form homodimers of Mw from 48 to 53 kDa through non-covalent bonds (Croy, Hoque, Gatehouse, & Boulter, 1984). In addition, a shoulder eluting at 19 min (Peak 1) appeared at pH > 4.0 with a high Mw of average 250 kDa. Samples at pH > 4.0 all showed similar elutions with a higher oligomeric fraction, also showed similar elution patterns, with

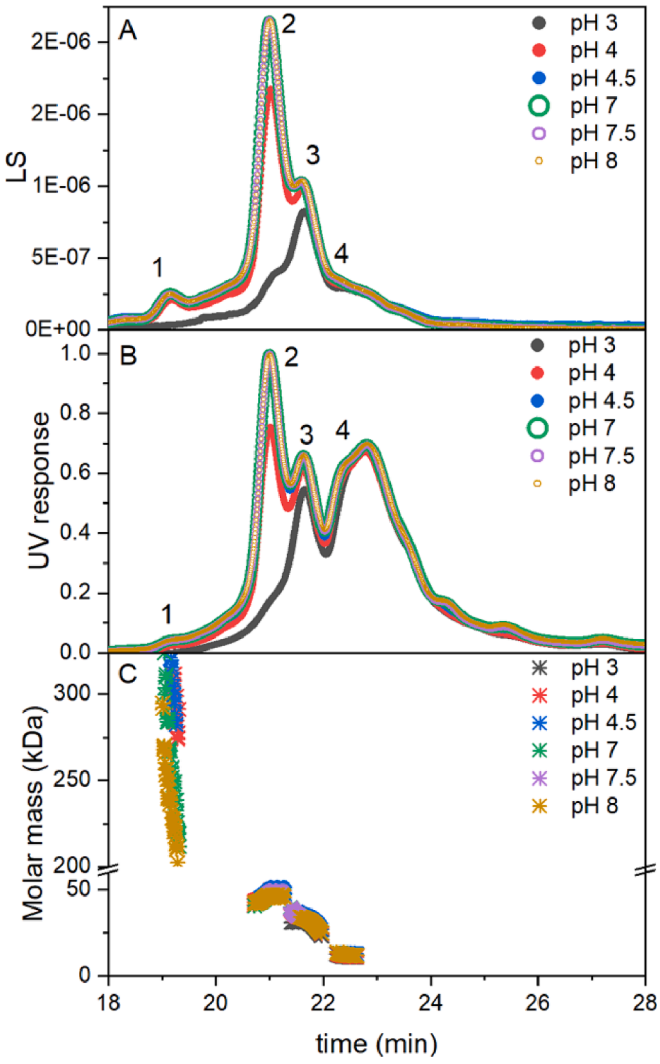

Fig. 2. SEC-MALS elution profiles with light scattering (LS) signal (A) and UV response (B), and molecular mass (C) of pea albumins solution (10 mg/ml) at different pH, from 3 to 8. All samples were eluted with the same elution buffer.

dimer and monomers, but also with the shoulder eluting earlier. It is important to note that all these samples were filtered before injection, and all aggregates were eluted at the same pH, and assuming that won't affect the size. While the population in the monomer and dimer peaks did not seem to change, at the higher pH there was a larger presence of aggregates. Also, it is important to note that all samples were injected at a comparable protein concentration (within experimental error).

Fig. 3 presents the SAXS intensity curves of pea albumins as a function of q for unheated solutions prepared at different pH values. The model fit for polydisperse spheres is also shown, and clearly, all treatments showed good fits, apart from the sample at pH 7, at low q range (<0.01 Å^{-1}). In this case, unlike for the SEC analysis, the samples were not filtered. As shown in Fig. 3, albumin solutions at pH 3, 7.5 and 8 showed similar scattering properties. There was no increase in the scattering at low q, indicating no aggregation. At the higher q range, the intensity of the pH 3 solutions was lower than for pH 7.5 and 8 solutions, which we attribute to a slightly different local shape. According to SEC-MALS data (Fig. 2), albumins at pH 7.5 and 8 also showed a small population of aggregates as well as dimers. It is important to note that sample comprised of different populations (Fig. 2), but based on the scattering signal, it may be suggested that the scatterers present may have local sizes of similar magnitude. More work is needed to obtain monodispersed populations, this would allow for a better understanding

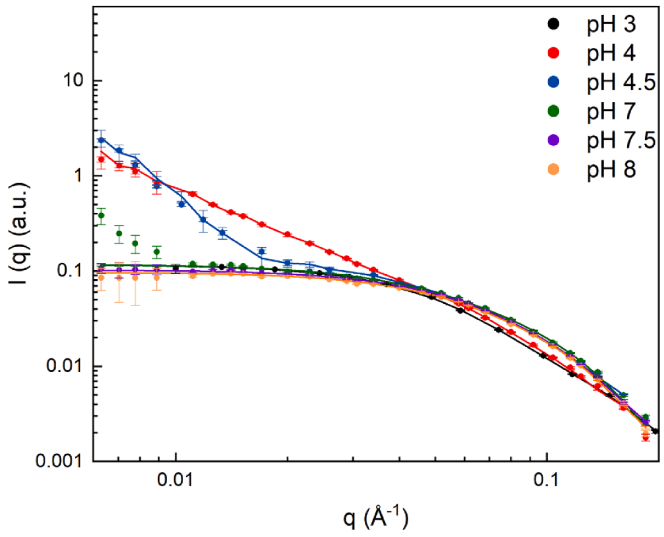

Fig. 3. Background-subtracted SAXS intensity for pea albumins solutions (10 mg/ml) as a function of pH, represented by the dot plot, lines representing model fits. Note: pH 3-black, pH 4-red, pH 4.5-blue, pH 7-green, pH 7.5-purple, pH 8-orange.

of their aggregation behavior with pH and heating.

As already shown in Fig. 2, albumins showed the presence of a dimer as well as oligomers at pH higher than 4. These samples were filtered before SEC, hence, larger aggregates have most likely not been analyzed. Indeed, SAXS scattering curves of solutions at 4, 4.5 and 7 showed much higher scattering intensities at low *q*. Fig. 3 clearly shows a marked increase in intensity at low *q* compared to the sample at pH 3. Furthermore, at pH 4.5 and 7, there were different upturns at low *q,* indicating significant differences in their structural features. The higher *q* upturn at pH 7 indicates a population of significantly larger aggregates than at pH 4.5. Furthermore, these aggregates were no longer noted at pH 7.5 and 8. These intermediate pH values, with an upturn in the scattering signal at low *q,* suggested substantial structural changes in solution for the albumin in the shift from low to high pH, likely due to the formation of non-covalent aggregates during a decrease in their ionic charge (Chen et al., 2022). At a higher pH these aggregates were less present.

The SAXS data, in combination with the SEC-MALLS clearly raise important questions on the structural reorganization of the various protein populations present in the albumin fraction, as a function of pH, and more research is needed to fully understand their dependence on pH, ionic strength, as well as the interactions with the other minor components (mostly carbohydrates) present in solution.

3.3. Structural changes of albumins using SAXS and SEC-MALS during heat treatment

Pea albumins showed a pH dependent effect with heating, as shown by their visual appearance after different heating times (0–5 min) at 90 °C (Fig. 4). Albumins solutions were still clear at pH 3, and 4, however, at all other pH they showed cloudy solutions and even a precipitate. Solutions at pH 4.5 formed soluble aggregates after 1 min, as shown by a cloudy solution. Extensive aggregation and precipitation were evident with heating at pH 7 and 7.5. Although the properties of the unheated soluble pea albumins were quite similar at pH 7.5 and 8.0, there was a significant difference in the aggregation between the two treatments. At pH 8.0 the aggregates were cloudy but did not show extensive precipitation, more precipitation was noted at pH 7.5 and 7. The differences in the heat-induced aggregation between pH 7 and 8 clearly suggested the importance of the increased charge repulsion at pH 8.

To better understand the differences in aggregation between the

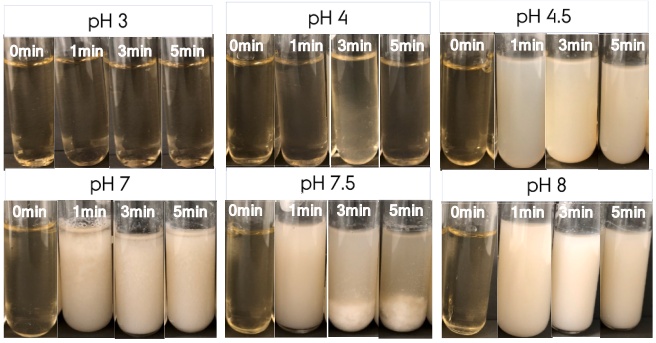

Fig. 4. Visual appearance of pea albumin solutions (10 mg/ml) after heating (0–5 min) at 90 °C at different pH values, from pH 3 to 8.

samples, the heated samples were analyzed by SEC-MALS, as shown in Fig. 5. In this case also, the solutions were filtered through 0.45 μm, hence only the residual native proteins, oligomers or intermediate aggregates could be analyzed here. Similarly to what is shown in Fig. 2 for unheated samples, 4 distinct peaks were also evident in the chromatographic elutions in the case of heated solutions, with important differences as a function of pH.

The UV absorption signal for albumins at pH 3 was unchanged with heating. There were only 2 peaks, corresponding to PA2 (peak 3) and PA1 (Peak 4) (Fig. 5A), as already described for the unheated samples. The lack of aggregation at pH 3 was in full agreement with their unchanged visual appearance with heating (Fig. 4). As shown in Fig. 5, with increasing pH from 4 to 8, peaks 1, 2, and 3 started to decrease in intensity, leaving only peak 4 nearly unchanged. Albumin solutions at pH 8 seemed to be more stable to heating compared to those at the lower pH values, from 4 to 7.5, as seen by a delay in peak 2 disappearance. Regardless, the visible differences in turbidity between the samples after heating (Fig. 4) would suggest that the aggregates were larger than several hundreds of nanometers, and therefore filtered out before SEC analysis. Comparing their Mw, in all cases there was a decrease in average mass with heating time, for the aggregates and oligomer peaks 1–3. These SEC results clearly indicated that PA2 dominated the aggregation, while the PA1-predominant peak was not altered, regardless of the heat treatment or pH.

The overview of background subtracted SAXS scattering patterns, for all albumin samples subjected to heat treatment as a function of pH is shown in Fig. 6. In addition, the particle size distribution determined using the polydisperse spheres scattering model for all treatments is presented in Fig. 7. The albumins at pH 3 showed no aggregation with heating; they only exhibited slight changes in scattering intensity, and their particle size histogram also only showed a slight shift to higher radii (Fig. 7). These results led to the conclusion that no heat-induced structural changes occurred at this pH, whereby the internal compact structure at pH 3 may have experienced a slight swelling, or limited aggregation.

Significant alterations of the scattering patterns with heating were evident for all the other pH treatments. At pH 4 the scattering pattern was quite different than for all the other pH values, indicating some residual local building blocks of spherical shapes, but also the presence of larger structures around 60–100 nm in radius (Fig. 7). Albumin solutions at pH 4.5, 7, 7.5 and 8, clearly showed the presence of small monomers and dimers, with a Guinier region shifting at higher *q* with heating and at the same time forming much larger aggregates as indicated by the steep increase in intensity at the low *q*. The high *q* features tended to be broader with increasing heating time (Fig. 6), on one hand, showing increased polydispersity, but also a gradual increase of the larger population leaving only the smaller population of protein particles. This could likely be the residual scattering from PA1, as SEC results would suggest. As shown in Fig. 7, there was a clear shift to larger, more

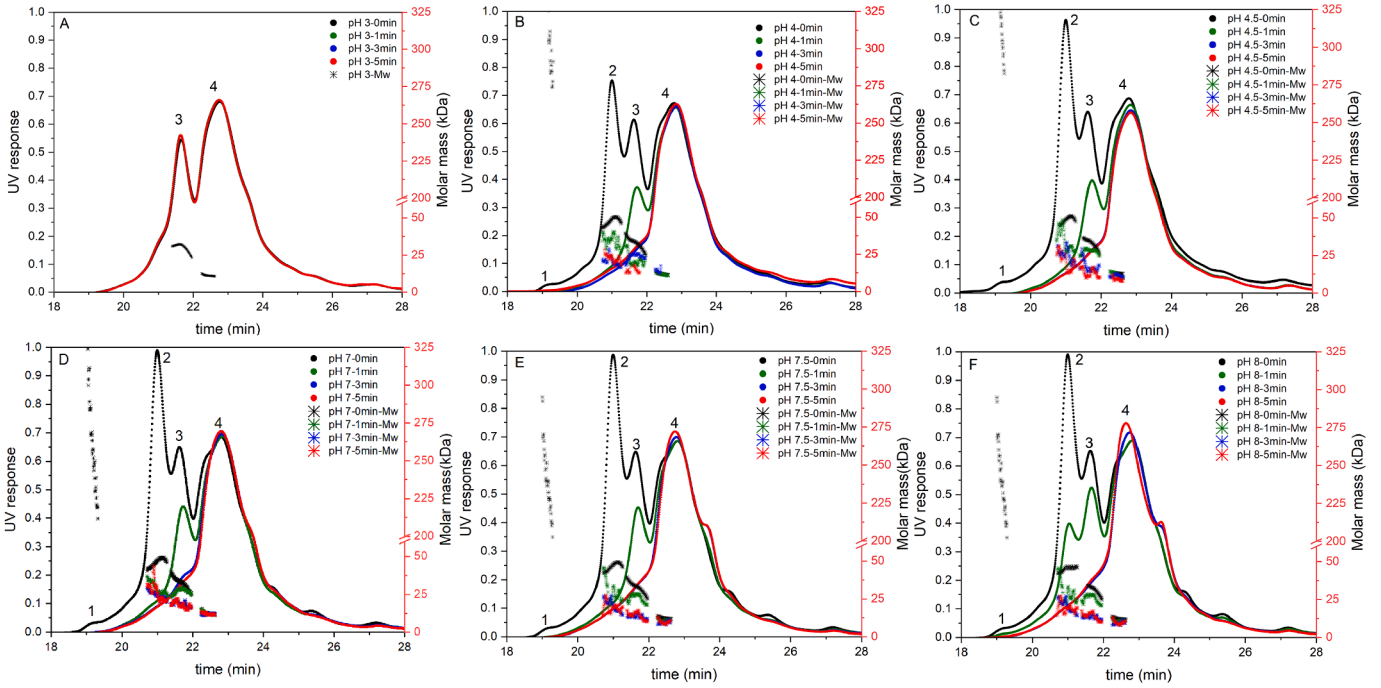

Fig. 5. SEC-MALS elution profiles (UV, left axis) including molecular weight of pea albumins solution (10 mg/ml) for different heating times (0–5 min) at 90 °C from pH 3 to 8 and corresponding molar mass (stars, right axis).

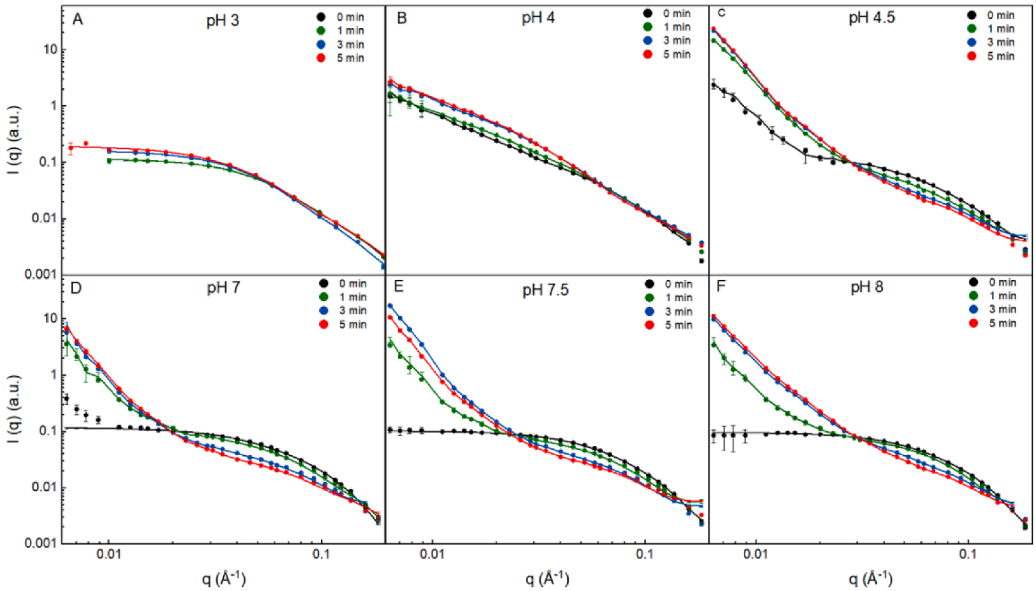

Fig. 6. Background-subtracted SAXS intensities (dots) of pea albumins solution (10 mg/ml) for different heating times (0–5 min) at 90 °C from pH 3 to 8, lines represent model fit.

polydisperse sizes with increased heating time. The intensity of the low q features increased (Fig. 6) and larger aggregates formed (Fig. 7) with longer heating times (3 and 5 min), confirming extensive protein aggregation (Chen et al., 2022; McEwan, Egorov, Ilavsky, Green, & Yang, 2011). The aggregates formed from Fig. 6 seemed to show a q dependence q^{-4} indicating the formation of compact and smooth aggregates (Chen et al., 2021; Liu et al., 2017), but with sizes beyond the limits of the SAXS measurement.

In summary, SEC combined with MALS and SAXS show the presence of some stable proteins, and the formation of oligomers and aggregates depending on the pH. A two steps aggregation is proposed, with small particles forming at the first step and then fractal structures organized by the small particles. This also agrees with the aggregation mechanism of whey proteins or β-lactoglobulin proposed previously during heating (Chen et al., 2022; Ikeda & Morris, 2002; Vijayalakshmi, Krishna, Sankaranarayanan, & Vijayan, 2008). The albumins could be modelled as spherical structures. At pH 3 the proteins showed a slight aggregation when subjected to heat treatment at 90 °C for 5 min. Albumins at pH 4 could also keep their spherical shape after heat treatment, however, in this case, some of the protein structure may have undergone unfolding and aggregation. The scattering intensity of the proteins at pH 4 increased and the aggregates became larger with increasing heating time, only from 0 to 3 min. However, at this pH, the strong charge repulsion force could limit the size of the protein clusters, by reducing

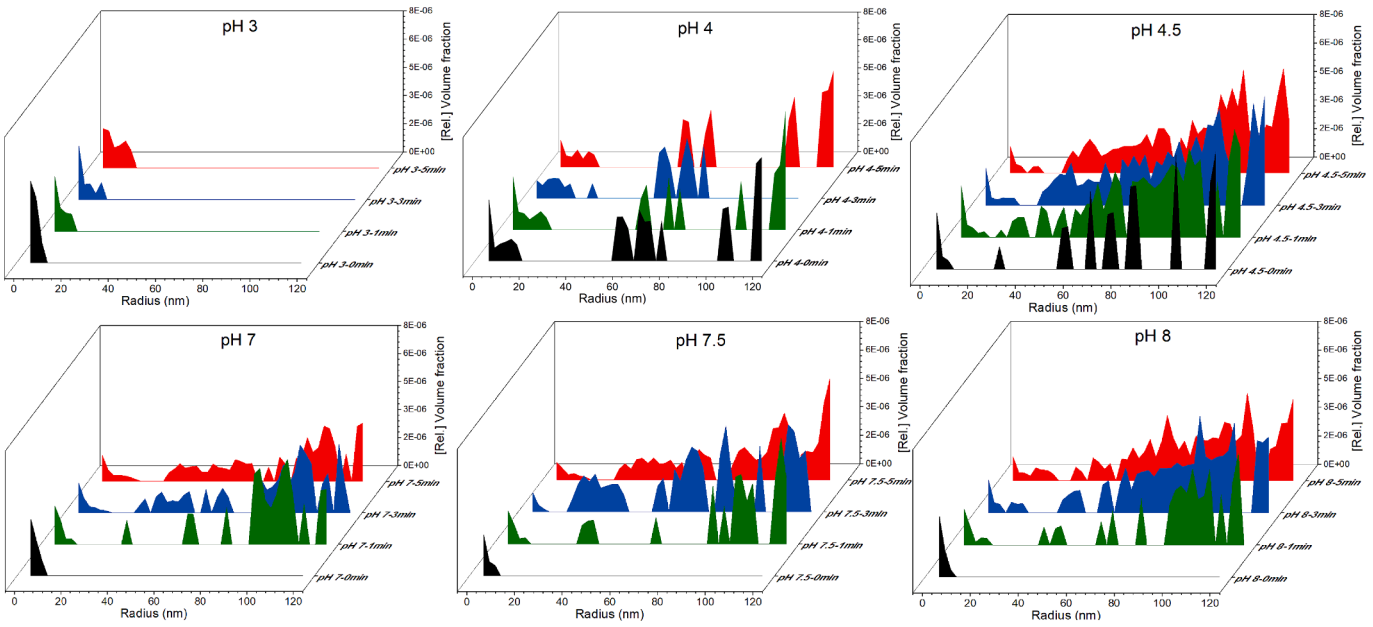

Fig. 7. Particle size distribution histograms of pea albumins solutions (10 mg/ml) after heating at different times (0–5 min) at 90 °C from pH 3 to 8, as estimated from the McSAS fit.

further aggregation at longer heating times (3–5 min). This has already been shown for other proteins (Chen et al., 2022; Nicolai, 2019). When pH increased to 4.5, the albumins became more sensitive to heat treatment, due to their much-decreased overall charge. More attachment points were assumed to be exposed and larger structures were formed probably via mainly non-covalent interactions with longer heating time, inducing a general disruption of their local structure. A similar behavior was also shown for samples adjusted at pH 7, 7.5 and 8. At this pH, the proteins are negatively charged, and may be much more sensitive to the presence of ions in the solution. The effect of ionic strength in the albumin aggregations was not within the scope of the current research.

4. Conclusions

The structural dynamics of a pea albumins in solution during heat treatment at various pH values were successfully followed using SAXS, combined with the measurement of molecular weight using SEC-MALS. While solutions at pH 3 showed low aggregation with heating at 90 °C for 1, 3 and 5 min, albumins close to the isoelectric point and at higher pH showed extensive aggregation. A model for polydisperse spheres was employed to estimate the changes in particle size distribution with heating at all pH values. The comparison of light scattering data from SEC and SAXS showed some discrepancies which may be due to sample preparation, namely, buffer re-equilibration and filtration. However, SEC data clearly suggested a conserved PA1 peak with the dimeric fraction, mostly composed of PA2, to be the most prone to heat-induced aggregation. However, further studies are needed to better understand the structural changes of pea albumin fractions under different processing conditions, and possibly starting with monodisperse fractions for a more detailed structural characterization. This work clearly highlighted the potential to use albumin proteins as ingredients in food, due to their process induced versatility in structure formation.

CRediT authorship contribution statement

Ruifen Li: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jacob J.K. Kirkensgaard:** Writing – review & editing, Validation, Supervision, Software, Methodology, Investigation. **Milena Corredig:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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