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Effect of pH and ionic strength on heat-induced pea protein isolate aggregation and gel formation

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ABSTRACT

Heat-induced aggregation and gelation of salt extracted pea protein isolates (PPI) was studied as a function of NaCl concentration (0.0–0.4 M) and pH (3.5–8.5). It was hypothesized that an isolate extracted with NaCl, and subsequently dialyzed would show different composition and techno-functional properties depending on its ionic environment. Protein solubility of PPI was affected by NaCl concentrations and pH, with the lowest solubility measured at pH 4.5, regardless of NaCl concentrations. At pH 3.5, solubility was high at low ionic strengths and decreased with increasing salt. At pH between 4.5 and 7, protein solubility increased in solutions at higher NaCl concentrations. At alkaline pH, where proteins are highly charged, salt concentrations did not affect solubility. Heating induced extensive protein aggregation in the presence of NaCl. However, in the case of heated samples in deionized water at pH 3.5 and 8.5, limited aggregation was noticed. These results were confirmed using atomic force microscopy on water redispersed samples. Analysis of viscoelastic properties at the least gelation concentration showed that pH and ionic strength affected not only the stiffness but also the linear viscoelastic regime. This work clearly demonstrated that solubility and thermal stability of PPI are affected by charge properties and how the structure and properties of pea protein aggregates may be modulated through careful control of pH and ionic environment, ultimately affecting the bulk properties of pea protein heat-induced gels.

1. Introduction

The growing demand for plant-derived protein products caused by environmental concerns over the overconsumption of animal-based products and increasing global population is driving the development of novel foods based on alternatives to animal proteins. Legume proteins, in particular, soy and pea proteins, are growing in demand as food ingredients due to their low cost, low impact on environment, high availability, and high acceptability. Plant protein-based gels, such as tofu, meat analogues, vegan jelly, and vegan desserts, are increasingly available in the markets. In the past decades, soy protein has captured two-thirds of the plant proteins market (ASA, 2024), with pea proteins being considered a good alternative to soy protein as an ingredient for food (Lam et al., 2018).

Pea proteins are usually categorized according to their solubility: salt-soluble globulins (55–65 %), water-soluble albumins (18–25 %), ethanol-soluble prolamins (4–5 %), and alkaline-soluble glutelins (3–4 %) (Lu et al., 2020). Globulins are the main storage protein and can be further classified into legumin, convicilin, and vicilin based on their different sedimentation coefficient. Pea legumin is a hexamer with a molecular weight (M_w) of about 360 kDa, with monomers (Mw~60 kDa) containing an acidic and a basic subunit. Pea convicilin is a trimer protein (Mw~210 kDa), composed of 3 monomers (Mw~70 kDa). Pea vicilin is the smallest globulin (Mw~150 kDa) and is a heterogeneous trimer with subunits of α (20 kDa), β (13 kDa), γ (12–16 kDa), and their combination of $\alpha+\beta$ (30–36 kDa) and $\beta+\gamma$ (25–30 kDa) (Tzitzikas et al., 2006).

The heat-induced gelation properties of proteins play a crucial role in food manufacture. The heat-induced gelation mechanism for globular proteins is commonly described in three overlapping stages. First, the compact globular structures of the proteins unfold, exposing the inside buried hydrophobic regions. Subsequently, the increased

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hydrophobicity, reactive cysteine and –SH residues induce intermolecular interactions, resulting in the formation of aggregates and extensive aggregation, which, at sufficient concentrations, will lead to the formation of a gel network (Guldiken et al., 2021; Tang et al., 2024). During heating, both soluble and insoluble aggregates may form (Klost et al., 2020). These aggregates are polydisperse, varying in composition and size, with some forming the networks while some acting as fillers within the protein network. The type of aggregates and their distribution will affect the properties of the gel, for example, the gel strength. A high distribution of insoluble aggregates may weaken the gel structure, leading to less stable gels with lower gel strength and water holding capacity (Klost et al., 2020); on the other hand, soluble aggregates may facilitate the formation of well distributed and interconnected protein networks (Ren et al., 2024).

It is established that the heat-induced formation of protein aggregates is influenced by various environmental factors such as pH, ionic strength, and temperature, leading to different types of gels (Gosal & Ross-Murphy, 2000; Guldiken et al., 2021). When the overall charge distribution of the protein is minimized, for example, with the pH close to the isoelectric point or at high ionic strength conditions, globular proteins form opaque, particulate gels due to the large size of the aggregates, while when the pH deviates from the isoelectric point and the electrostatic repulsion is strong, fine stranded transparent gels may form, consisting of flexible intermediate aggregates (Nicolai & Durand, 2013). However, the effect of salt on the aggregation and gel properties of pea protein isolate has yet to be studied in detail. The stiffness of gels made from pea protein isolate obtained through salt extraction and micellization was the highest at pH 4.5, and the lowest at pH 9, irrespectively of ionic strengths (Tanger et al., 2022). On the other hand, the gel stiffness at pH 3 increased with increasing ionic strength (0–0.5 M NaCl), while at pH 7 displayed an opposite trend (Tanger et al., 2022). The presence of 0.5 M NaCl was also reported to reduce the least gelation concentration of pea protein isolate suspensions at pH 7 (Guldiken et al., 2021). The inconsistencies reported in literature possibly derive from differences in the protein purification history, protein composition of the isolates, and ionic environment. Indeed, the ratio between the various globulins, and in particular, legumin to vicilin, influences the properties of the gel (Guldiken et al., 2021). This ratio is affected by ionic environment (Zhang & Corredig, 2023). Furthermore, with heating, pea legumins were reported to tend to form large, insoluble aggregates, detrimental to gel formation, while pea protein isolates with a higher ratio of vicilins can form stiffer gels due to the smaller intermediate aggregates formed during heating (Nicolai & Chassenieux, 2019).

Even though extensive studies have investigated the effect of pH and/or ionic strength on protein aggregation and/or gel properties (Klost et al., 2020; Sun & Arntfield, 2011; Tanger et al., 2022), limited studies have systematically investigated the effect of ionic strength at various pH levels. Sun and Arntfield (2011) studied the effect of pH and salt on pea protein gelation properties, while they didn't look at the protein aggregation behaviors. Recently, Tanger et al. (2022) investigated the influence of both pH and ionic strength on the solubility and thermal gelation behaviors of pea protein. However, the effects of salt and pH on the solubility and aggregation behavior of pea protein at room temperature is not enough to explain the properties of the heat-induced gel. As the solubility change during heating is also important to investigate the pea protein aggregation behavior during this process. To really understand the gelation behaviors of resulting heat-induced gels, a detailed and systematic study on the relationship between the effect of pH and ionic strength on the aggregation of pea protein isolates before and after heating and the consequent influence on the properties of the gels formed is needed. To fill this gap, a pea protein isolate was prepared by salt extraction, extensively dialyzed, and the effect of processing conditions (pH and NaCl concentration) on pea protein aggregation both before and after heating was investigated in relation to the properties of the heat-induced gels formed.

2. Materials and methods

2.1. Materials

Pea protein concentrate (PPC, 49 % protein, w/w, dry basis) was obtained from Vestkorn A/S (Holstebro, Denmark) using air classification. Distilled water was used throughout the study. Sodium chloride (NaCl, ≥99.5 %) was obtained from VWR (USA). Sodium hydroxide (NaOH, ≥97 %) was purchased from Sigma-Aldrich (USA). Hydrochloric acid (HCl, 37 %) was sourced from Fisher Scientific (USA). All other chemicals were of analytical grade and purchased from regular suppliers.

2.2. Pea protein isolate preparation

To prepare the pea protein isolate (PPI), the pea protein concentrate (10 % w/v) was dispersed in 0.4 M NaCl, and pH adjusted to 7.0 with 1 M NaOH, and stirred for 2 h while maintaining the pH. The suspension was centrifuged (4500 *g*, 4 °C, 20 min), and then the supernatant was dialyzed, lyophilized, and kept at −20 °C for further analysis.

The protein content was determined using the Dumas combustion method (DUMATHERM®, Gerhardt Analytical Systems, Germany) with a nitrogen conversion factor of 5.4 (Mariotti et al., 2008). Ash content in extracted pea protein was determined by measuring the residual weight after heating the sample in the muffle furnace (Nabertherm, Germany) at 550 °C for 5 h. The crude protein content of the salt extracted PPI after freeze drying was 68.8 % (based on 5.4 conversion factor) and the ash content was 4.06 %.

2.3. Heating of PPI suspensions

Dry samples were re-solubilized in different NaCl solutions (0.0 M, 0.05 M, 0.1 M, 0.4 M), adjusted to different pH values (3.5, 4.5, 6.5, 7, 8.5), for each NaCl concentration, to a final constant protein concentration of 2 mg/mL. The mixtures were vigorously stirred for 1 h at room temperature (22 °C) and aliquots (5 mL) were transferred to a glass tube, sealed and then subjected to heat treatment (95 °C, 1 h) in a water bath. Their unheated counterpart was kept at room temperature (22 °C).

2.4. Solubility and turbidity

Solubility of the PPI suspensions was defined by measuring the difference in protein concentration of a 1 mL sample after centrifuging (Sorvall™ ST 8 Small Benchtop Centrifuge, Thermo Scientific, US) at 4500 *g*, 4 °C, 20 min. In addition, before centrifugation, photographs were taken to document differences in appearance and their turbidity was tested by measuring UV absorbance at 600 nm with a Thermo Scientific™ Multiskan SkyHigh Microplate Spectrophotometer (Thermo Scientific, US) using a disposable plastic cuvette. The protein concentration in the supernatant was measured using BCA kit (Pierce™ BCA Protein Assay Kits, Thermo Scientific, US) measuring absorbance at 562 nm using an Eon™ Microplate Spectrophotometer (Eon Biotech PTE. LTD, Singapore).

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE analysis was conducted to evaluate the soluble protein composition in sample supernatants after dispersing the PPI in different NaCl concentrations (0.0 M, 0.1 M, and 0.4 M; 0.05 M was excluded due to its solubility and aggregation behavior being similar to 0.1 M) and at different pH (3.5, 4.5, 6.5, 8.5), stirring for 1 h, and following centrifugation at 4500*g*, 22 °C, 20 min (Sorvall™ ST 8 Small Benchtop Centrifuge, Thermo Scientific, US). Aliquots (60 μL) of the supernatants were mixed with NuPAGE LDS sample buffer (20 μL) and then loaded (10 μL) to the NuPAGE™ (4 − 12 %, gradient) Bis-Tris

precast mini protein gels fixed in the Invitrogen™ XCell SureLock™ Mini-Cell, running in NuPAGE™ MES SDS running buffer with a voltage of 200 V for 35 min. The gels were fixed in a solution containing 50 % ethanol, 8 % phosphoric acid for 2 h before staining with Coomassie blue (5 % w/v Al-sulfate, 2.35 % v/v Phosphoric acid (85 %), 0.02 % v/v Coomassie blue, and 10 % v/v Ethanol (96 %) in MilliQ water) for 2 h. Then the gel was washed in water for 1.5 h and subjected to the image analysis Gel Doc™ EZ System (Image Lab™, BIO-RAD laboratories, USA).

2.6. Circular dichroism spectroscopy (CD)

CD measurements were conducted to investigate differences in the secondary structure of the PPI solutions after centrifugation, according to the method reported by Herneke et al. (2021), using a Jasco J-810 spectropolarimeter (Jasco Corp., Japan). After resuspension at different pH (3.5, 4.5, 6.5, 8.5) and NaCl concentrations (0.0 M, 0.05 M, 0.1 M, 0.4 M), followed by centrifugation at 4500*g*, 4 °C, 20 min (Sorvall™ ST 8 Small Benchtop Centrifuge, Thermo Scientific, US), the protein concentrations of all supernatant samples were diluted to 0.1 mg/mL with 10 mM phosphate buffer (pH 7). Protein concentration was measured using BCA kit. The samples were measured in the range of 180–250 nm using a quartz cuvette with a path length of 1 mm. Phosphate buffer was used as background.

2.7. Atomic force microscopy (AFM)

Selected samples were characterized by evaluating their surface morphology using AFM, according to the method described by Herneke et al. (2023) using the Bruker Dimension FastScan instrument (Bruker, US). PPI samples in water (2 mg protein/mL) with different pH values (3.5, 4.5, 6.5, 8.5) were observed before and after heat treatment (95 °C, 1 h), after diluting 100–500 times with water. A drop (15 μL) of the diluted samples was loaded to the mica plate and air dried. FastScan B cantilevers (Bruker, US) were used in this study and images were processed using NanoScope Analysis and Gwyddion 2.43.

2.8. Least gelation concentration (LGC)

Least gelation concentration was measured according to the method reported by Coffmann and Garciaj (1977). Sample suspensions of pea protein treated with different salt and pH were prepared in the protein concentration range of 10–120 mg/mL. After heating in a glass tube at 95 °C water bath for 1 h, the samples were put in room temperature to cool down for 4 h. The least gelation concentration was determined as the lowest concentration at which a self-supporting gel formed, remaining stable without slipping or falling when the tubes were inverted. Due to sample conservation, we decided to cut the 0.05 M concentration, as there was a similar solubility and aggregation behavior of PPI in 0.05 M and 0.1 M NaCl solutions. Thus, 0.0 M, 0.1 M, and 0.4 M NaCl were used for the LGC and following rheology test.

2.9. Rheological properties of pea protein gels

Gels formed, at the least gelation concentration were carefully spooned out and placed between a 40 mm parallel plate geometry with a gap of 1.5 mm. Rheological analysis was performed by applying a frequency sweep (oscillation strain 0.5 %, frequency 0.01–10 Hz) and a strain sweep (frequency 1 Hz, oscillation strain 0.1–1000 %), using a DHR-20 rheometer (TA Instruments, US). The critical strain γ_c was defined as the strain point where storage modulus G' showed a change of at least 5 % from the value in the linear viscoelastic regime, or plateau value (Yang et al., 2021).

2.10. Statistical analysis

All results were obtained from two independent replicates and presented as the mean ± standard error. The difference between the mean values were considered significant within a 95 % confidence interval using one-way ANOVA analysis and Duncan test, using SPSS 20 software (SPSS Inc, Chicago, IL, USA). A Paired-samples T test was applied to determine the significant difference between paired groups ($P < 0.05$).

3. Results and discussion

3.1. Turbidity

It is known that the net charge of proteins affects their solubility, intermolecular interactions, and aggregate morphology (Nicolai & Durand, 2013). Hence, the pH and ionic strength of pea protein solutions was studied for their solubility and the presence of aggregates. The visual appearance of the PPI solutions at different pHs and NaCl concentrations before and after heat treatment is shown in Fig. 1A, together with the corresponding turbidity results (Fig. 1B–C). PPI solutions at pH 4.5 were the most turbid regardless of ionic strength and showed the highest extent of precipitation after heating (Fig. 1A). The extensive aggregation was due to decreased electrostatic repulsion close to the proteins' isoelectric point (Salis et al., 2011). At pH values away from this pH, proteins exhibited stronger electrostatic repulsion, leading to decreased aggregation and lower turbidity (Fig. 1B). This effect was particularly pronounced at alkaline conditions (pH 8.5), where proteins are negatively charged, and unheated solutions appeared transparent (Fig. 1A). Interestingly, the solutions at low pH (3.5) showed higher turbidity than at pH 8.5, which could suggest a potential complexation with negatively charged impurities still present in the PPI, namely phytates or polygalacturonic acid-containing carbohydrate chains.

The presence of salt influenced the formation of aggregates, as clearly noted at pH 3.5 and 6.5, in both heated and unheated samples (Fig. 1B–C). Within a certain pH value, there was little difference in the appearance and turbidity for concentrations from 0.0 to 0.1 M NaCl. However, at 0.4 M NaCl concentration, samples at pH 3.5 were the most turbid, while the lowest turbidity was measured for PPI suspensions at pH 6.5 (Fig. 1B).

The turbidity of PPI suspensions in the pH range between 4.5 and 8.5 with different salt concentrations (0.05–0.4 M NaCl) increased after heating (Fig. 1C). With higher salt concentrations, there was higher turbidity, indicating salt enhanced heat-induced pea protein aggregation. These results confirmed previous observations in soy protein isolate suspensions (Jiang et al., 2010). Interestingly, at low pH (pH 3.5), the turbidity of PPI in different salt solutions was not influenced by the extensive heat treatment used in this work (95 °C for 1 h) (Fig. 1B–C). Similar effects of heating on pea protein isolates at low pH value have been reported (Munialo et al., 2014). It was then possible to conclude that the aggregation of pea protein isolates can be controlled by environmental conditions, and especially when maintaining a high overall charge of the proteins and low ionic strength during the heating process.

3.2. Protein solubility affected by NaCl, pH, and heat

Protein solubility is crucial for effective techno-functional properties like emulsification, foaming, and gelation (Tanger et al., 2022). During heating, the amount of soluble proteins can influence protein interactions and the formation of soluble aggregates, ultimately affecting the elasticity and deformation properties of heat-set gels (Shand et al., 2007). The changes in PPI solubility depending on NaCl concentration and pH, before and after extensive heating at 95 °C for 1 h are shown in Fig. 2. In general, the solubility results are consistent with the turbidity measurements (Fig. 1B). The addition of salt reduced protein solubility at low pH but enhanced the solubility at and above the isoelectric point.

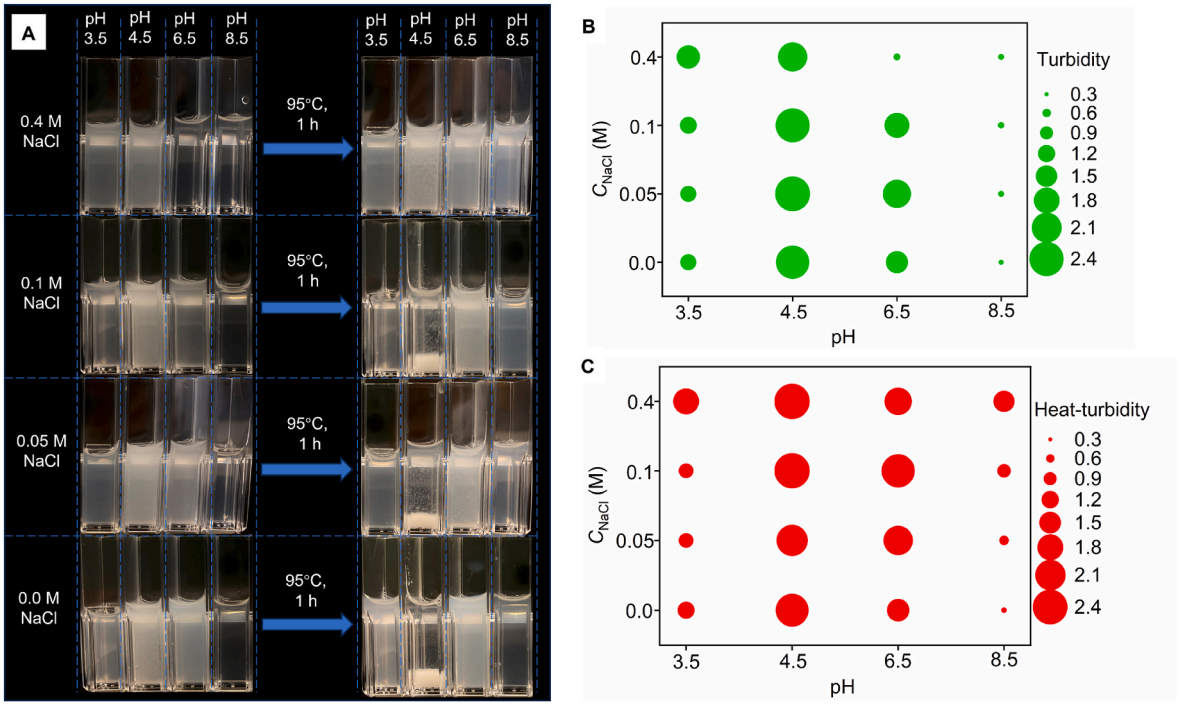

Fig. 1. Visual appearance (A) and turbidity of PPI (2 mg protein/mL) as a function of pH (3.5, 4.5, 6.5, and 8.5) and with different NaCl concentrations (0.0, 0.05, 0.1, and 0.4 M) under unheated (B) and heated (95 °C, 1 h) (C) conditions.

This phenomenon has also been reported in previous studies (Johansson et al., 2023; Mcwatters & Holmes, 1979). According to Salis et al. (2011), salt can shift the protein isoelectric point to a lower pH, thereby influencing the solubility. For the non-heated PPI (Fig. 2A), it showed the lowest solubility at pH 4.5. As the pH moved further away from the isoelectric point of proteins, solubility increased, reaching its maximum at pH 8.5, as extensively reported in the literature (Jiang et al., 2017; Shand et al., 2007). Within the pH range of 4.5–7.0, protein solubility showed a similar increasing trend with rising pH, though variations were observed depending on NaCl concentration: the solubility decreased at low ion strength (0.05 and 0.1 M NaCl) and increased at 0.4 M, because of salting-in effects. With higher salt concentrations, there is an increased hydration of the protein moieties, thereby increasing protein solubility (Arakawa & Timasheff, 1982). This behavior has been also reported for soy conglycinin, where solubility decreases at low salt concentrations of 0.05 M and 0.1 M NaCl, and then increases with increasing NaCl concentration up to 0.7 M (Zhang et al., 2022). PPI solutions in water at pH 7 had a relatively high solubility of 60 %, confirming an earlier report by Tanger et al. (2020). Salt extraction is considered a mild method compared to isoelectric precipitation, as it avoids extreme pH adjustments that could potentially denature the proteins and cause irreversible aggregation. Furthermore, pea globulins have an isoelectric point of pH 4.5, thus, they dominate the protein composition in the isoelectric precipitated PPI. In contrast, the salt extracted and dialyzed isolate used in this study contained both albumin and globulins (Fig. 3).

No significant changes were observed in solubility after heating when PPI was dissolved in deionized water at the pH range of 3.5–8.5, except for solutions prepared at pH 4.5 where the solubility decreased from 25 % to 16 % after heating (Fig. 2B). This is an interesting observation, which would suggest that the PPI extracted at 0.4 M NaCl and subsequently dialyzed not only maintained its solubility, despite the low ionic strength, but also showed to be stable to heat treatment. This result is consistent with previous report on pea protein by Chao and Aluko (2018), who found that heating (100 °C) had no influence on the solubility of the isoelectric precipitated PPI in water at pH 3–7, while it decreased the protein solubility at pH 7–9. On the other hand, the solubility of PPI solutions dissolved with 0.4 M NaCl, significantly decreased after heating regardless of pH. In general, the presence of salt enhanced protein aggregation and precipitation during the heating process. The decrease in PPI solubility with salt addition after heating can be attributed to several factors. First, the presence of NaCl facilitated the protein unfolding during heating (Du et al., 2017; Zhang & Corredig, 2023). Second, while salt enhanced protein solubility before heating, it also increased the possibility of hydrophobic interaction between protein residues exposed during the heat-induced unfolding of pea protein. These interactions resulted in extensive protein aggregation and subsequent precipitation, ultimately leading to the observed decrease in solubility.

3.3. *Soluble protein composition affected by NaCl and pH*

Different NaCl and pH environments led to variations in the protein composition of the soluble fraction of PPI dispersions. The protein profiles of supernatants from solubility test were analyzed using SDS-PAGE under non-reducing condition, as shown in Fig. 3. The band at 60 kDa can be attributed to the legumin monomer, which consists of an acidic and basic subunit with molecular weight (Mw) of 40 and 20 kDa, respectively (Lu et al., 2020). Band at 70 kDa represents the convicilin (Emkani et al., 2021). The band around 100 kDa was identified as lipoxygenase (LOX), a protein naturally present in pea seeds that was co-extracted during the PPI extraction process. The band at 55 kDa was identified as vicilin monomer, and the bands at 13–19 and 35 kDa were linked to the dissociated subunits of vicilin (Dziuba et al., 2014). Bands at 28 and 10 kDa can be attributed to albumin, present in all soluble fractions, due to the salt extraction process.

PPI supernatants of suspensions at different pH values displayed similar band distributions, and in general, bands intensity of globulin fractions increased with increasing pH from 3.5 to 8.5, except the samples at pH 4.5. This is consistent with the solubility results. At pH 4.5, the protein composition varied with ionic strength: only albumin, and the legumin basic subunit were found in PPI at pH 4.5 in 0.0 M NaCl. At 0.1 M NaCl, also the acidic subunit of legumin and vicilin were present in the soluble fraction, and at higher salt concentrations, for

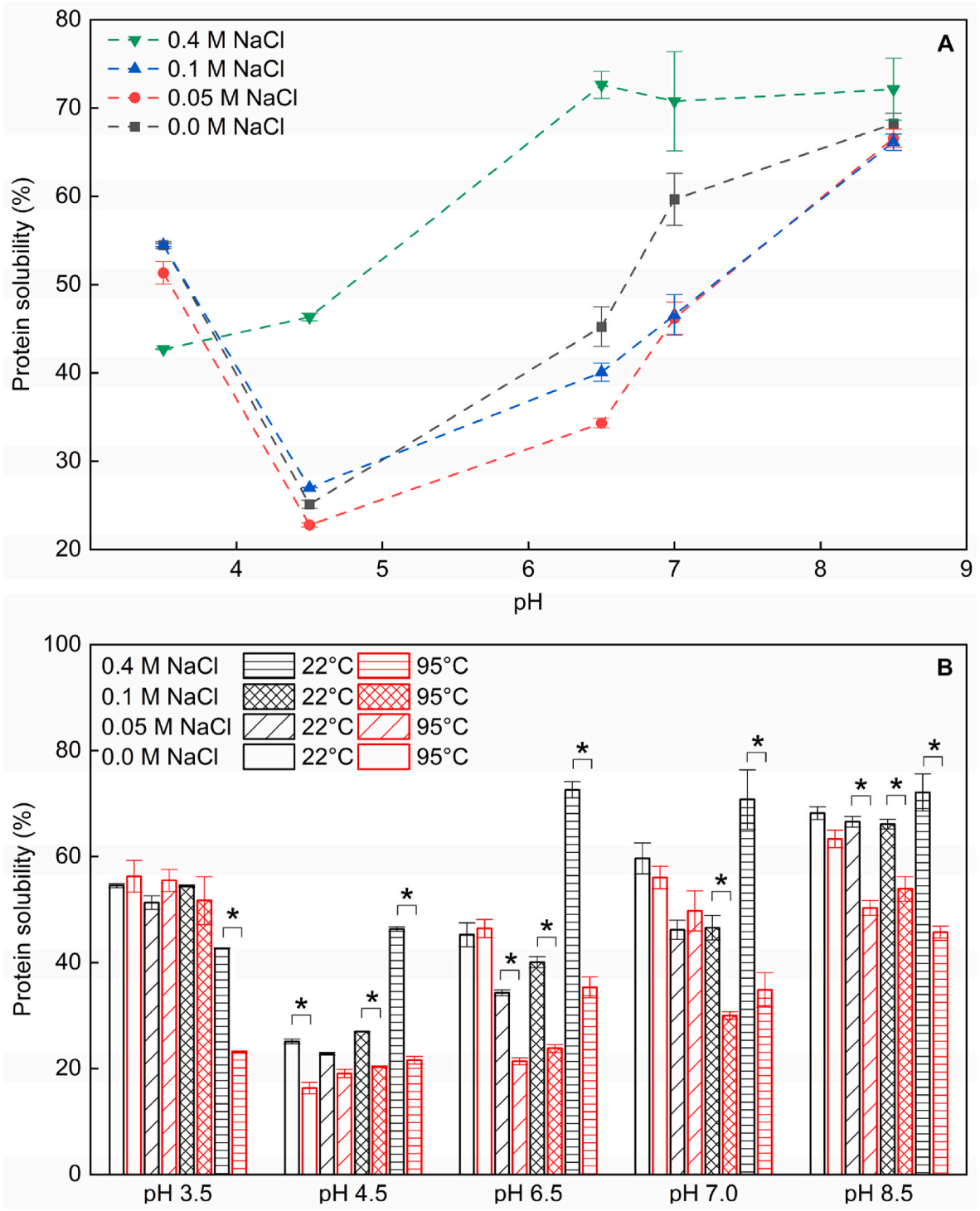

Fig. 2. Protein solubility of PPI dispersions (2 mg protein/mL) as a function of pH (3.5, 4.5, 6.5, 7.0, and 8.5) in NaCl solutions (0.0, 0.05, 0.1, and 0.4 M NaCl) (A), and solubility before (black bars, 22 °C) and after heating at 95 °C for 1 h (red bars) (B). * indicates significant pairwise differences ($P < 0.05$). Values represent the mean, and error bars represent the standard error (n = 2).

example, 0.4 M NaCl, more vicilin monomer and subunits, convicilin, and legumin acidic were solubilized. It was observed that the band intensity of the legumin monomer in 0.4 M NaCl was stronger at pH 3.5 than at pH 4.5, while the solubility of PPI in 0.4 M NaCl was lower at pH 3.5 compared to pH 4.5. Simultaneously, the intensities of some other bands were weaker at pH 3.5 than at pH 4.5, such as the band corresponding to the subunit with a molecular weight of 100–120 kDa. Their band intensities decreased with increasing NaCl concentrations at pH 3.5 while increased at pH 4.5, which may explain the lower solubility of PPI in 0.4 M NaCl at pH 3.5 than at pH 4.5. However, at other pH levels, the salt concentration had no obvious influence on the protein composition. These results point to an important difference between the role of salt during extraction of pea protein, and the role played in enhancing solubility.

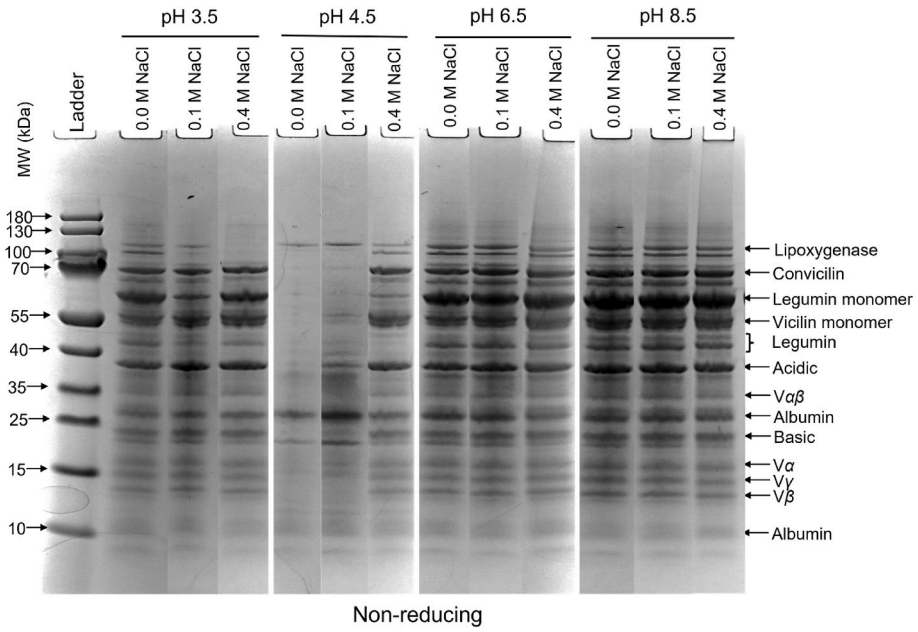

Fig. 3. SDS-PAGE protein profiles of soluble fractions of PPI dissolved with different concentrations (0, 0.1, and 0.4 M) of NaCl, at pH 3.5, 4.5, 6.5, and 8.5, under non-reducing condition. Acidic is the legumin acid subunit and Basic is the legumin basic subunit. V_{α}, V_{β}, V_{γ}, and $V_{\alpha\beta}$ are the different subunits of vicilin.

3.4. Atomic force microscopy (AFM)

To better evaluate potential differences in the aggregates' morphology of PPI solutions at low ionic strength and different pH values, AFM was employed to analyze samples before and after heating (Fig. 4). Near the isoelectric point at pH 4.5, micrometer size aggregates were observed. Shifting the pH away from the isoelectric point effectively reduced the size of protein aggregates. Solutions at pH 8.5 showed the smallest sizes, with pea proteins present as 30–40 nm spherical assemblies. Upon heating at 95 °C for 1 h, the large aggregates at pH 4.5 showed large clusters; this was also evident at pH 6.5. Protein solutions at pH 8.5 were less affected by the heat treatment, however, still showed the presence of aggregates, but much smaller than those formed with heating at pH 4.5 and pH 6.5. In contrast, heating of pea protein solutions at pH 3.5 reduced the protein aggregate size. Suspensions containing NaCl were not studied due to the significant interference of salt crystals deposited on the mica plate after drying.

3.5. Structural changes of PPI

Circular dichroism (CD) was employed to evaluate the structural changes occurring to the soluble fractions as a function of pH and NaCl.

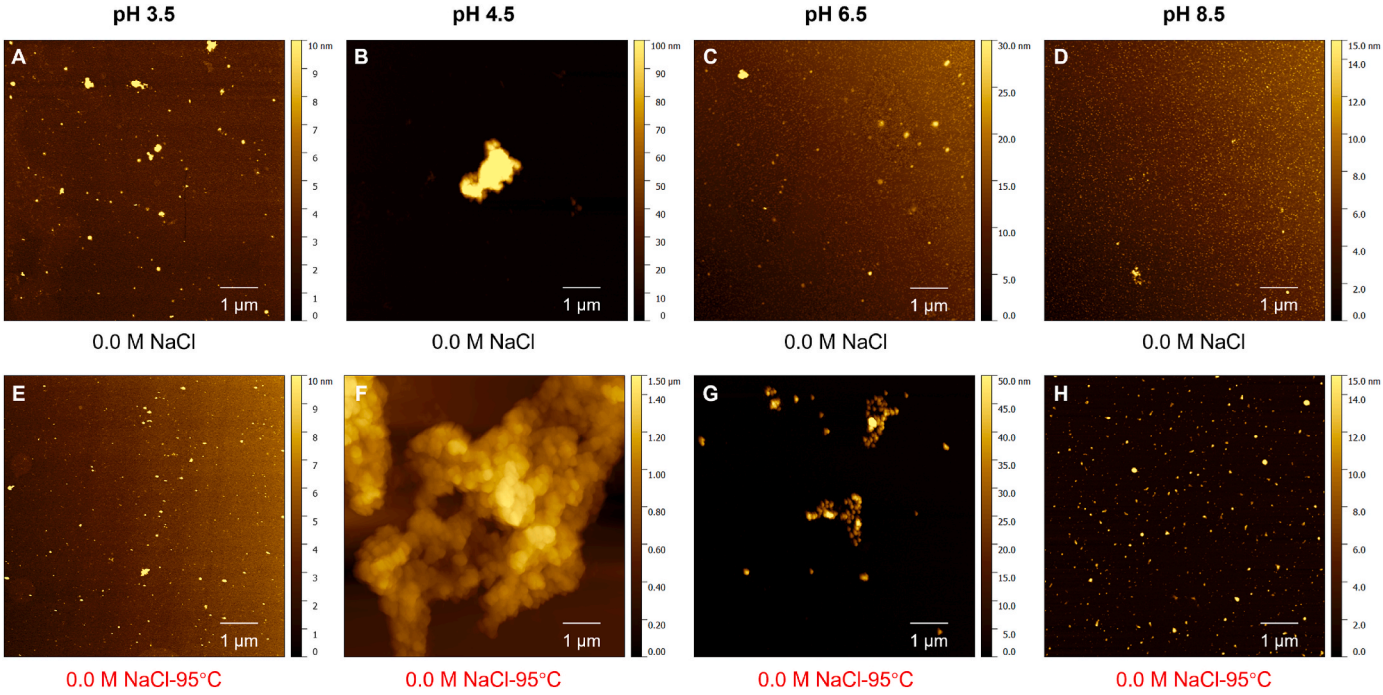

Fig. 4. Atomic force microscopy images of PPI as a function of pH (3.5 (A, E), 4.5 (B, F), 6.5 (C, G), and 8.5 (D, H)) dissolved in deionized water (0.0 M NaCl) before (top, black label) and after (bottom, red label) heating at 95 °C, 1 h.

The proteins' solutions, after reconstitution, as well as after heating, were centrifuged and diluted to 0.1 mg/mL with phosphate buffer at pH 7. By diluting at pH 7 in phosphate buffer, it was possible to assess any irreversible structural change occurring to the proteins. Proteins showed distinct CD spectra patterns, originating from their secondary structures. For example, a protein rich in α-helix structures would show two negative bands at 222 nm and 208 nm and one positive band at 190 nm in CD spectrum; in turn, β-sheet would provide signals with a negative band at 218 nm and a positive band at 195 nm; finally, disordered structures would display a negative band around 195 nm (Greenfield, 2007; Tiong et al., 2024). The measured CD spectra of the various pea protein solutions, with different pH and NaCl are shown in Fig. 5. PPI solutions at pH 3.5 (Fig.s. 5A), 6.5 (Fig. 5C), and 8.5 (Fig. 5D) displayed similar CD spectra, regardless of ionic strength in the range between 0.0 and 0.4 M NaCl. The spectra showed a positive peak around 195 nm, negative peaks in the range of 208–222 nm, and a zero crossing at 202 nm, suggesting structural similarities, mostly deriving from α-helix and β-sheets features. This result can be explained by their composition, mostly containing pea globulins, as shown in Fig. 3. In contrast, the CD spectra of soluble PPI at pH 4.5 (Fig. 5B) exhibited a different secondary structure compared to that of the other pH values and changed with salt concentrations. For PPI solutions at pH 4.5 without salt, containing predominantly albumins, the spectra exhibited a minor positive peak around 190 nm and an obvious negative peak 205 nm, suggesting the presence of a disordered structure with a minor presence of α-helix, from the original albumin structure. With increasing salt concentrations, more globulins were solubilized, and the spectra showed both positive and negative peaks shifts with an increased relative ratio of intensity between the positive and negative peak. This change indicated a higher presence of α-helix and β-sheets. At 0.4 M NaCl, the proteins at pH 4.5, containing more globulins, exhibited a positive peak around 196 nm and two negative peaks at 208 and 222 nm, suggesting structural similarities to those at the other pH values.

Heating at 95 °C for 1 h had no influence on the secondary structure of the soluble PPI in deionized water, at different pH values, except for at pH 4.5. For the heated soluble PPI fractions at pH 3.5 (Fig. 5E) with lower concentrations of salt (0.05 M and 0.1 M NaCl), their spectra exhibited minor positive peak and left shift of the negative peak, indicating a transition from order to disorder structure after heating. Furthermore, at this pH and 0.4 M NaCl, the secondary structure changed extensively, with the positive peak diminished, accompanied by an intensified negative peak at 202 nm, indicating the loss of structure. Similar disordered structures were found in soluble pea protein at pH 4.5 (Fig. 5F) at different concentrations of salt after heat treatment. For the heated soluble PPI fractions at pH 6.5 (Fig. 5G) with different concentrations of salt (0.05–0.4 M NaCl), their spectra exhibited similar disordered structure. At pH 8.5, increasing salt concentration after heating (Fig. 5H) exhibited a reduced positive peak and left shift of negative peak, for the soluble protein fraction indicating increased unfolding of the secondary structure.

3.6. Least gelation concentrations (LGC)

The least gelation concentration (LGC) is widely recognized as an indicator of the protein gelation capacity. Lower LGC values suggest better gelling abilities to form self-supporting network after heating (Boye et al., 2010; Ge et al., 2021). LGC of pea protein solutions suspended at different NaCl concentrations and pH values are shown in Fig. 6. The LGC of pea protein concentrate in water is 100 mg/mL (Fig. 6A), other researchers have reported similar results (Zhu et al., 2022). Different pH and NaCl concentration significantly influenced the LGC of PPI (Fig. 6B), with the highest LGC value for pH 8.5. At pH 6.5, pea proteins showed the lowest LGC, at about 20–40 mg/mL. The lowest LGC of PPI (20–40 mg/mL) is lower than LGC of any reported literature on isoelectric precipitated pea protein (Ge et al., 2021; Sun & Arntfield, 2010; Yang et al., 2021), possibly due to that pea protein extracted with NaCl, with no extreme pH adjustment, maintains its gelation functionality. Yang et al. (2021) reported a lower LGC (120 mg/mL) of salt extracted and dialyzed pea protein isolate than that of isoelectric precipitated pea protein (160 mg/mL), indicating the stronger gelation capacity of pea protein isolated by salt extraction followed by dialysis. Sun and Arntfield (2010) also reported a minimum gelation concentration of 55 mg/mL for salt extracted pea protein isolate, much lower than that of commercial pea protein isolate with a LGC of 145 mg/mL. Furthermore, literature values are, overall, much higher than those reported in the present study (Chen & Campanella, 2022; Sun & Arntfield, 2010; Wong et al., 2013).

Fig. 6B also shows the effect of salt addition on LGC. For the pea proteins at pH 3.5 and 6.5, the LGC values decreased from 80 to 40 mg/mL in water to 40 and 20 mg/mL in 0.1 M NaCl, respectively. Further

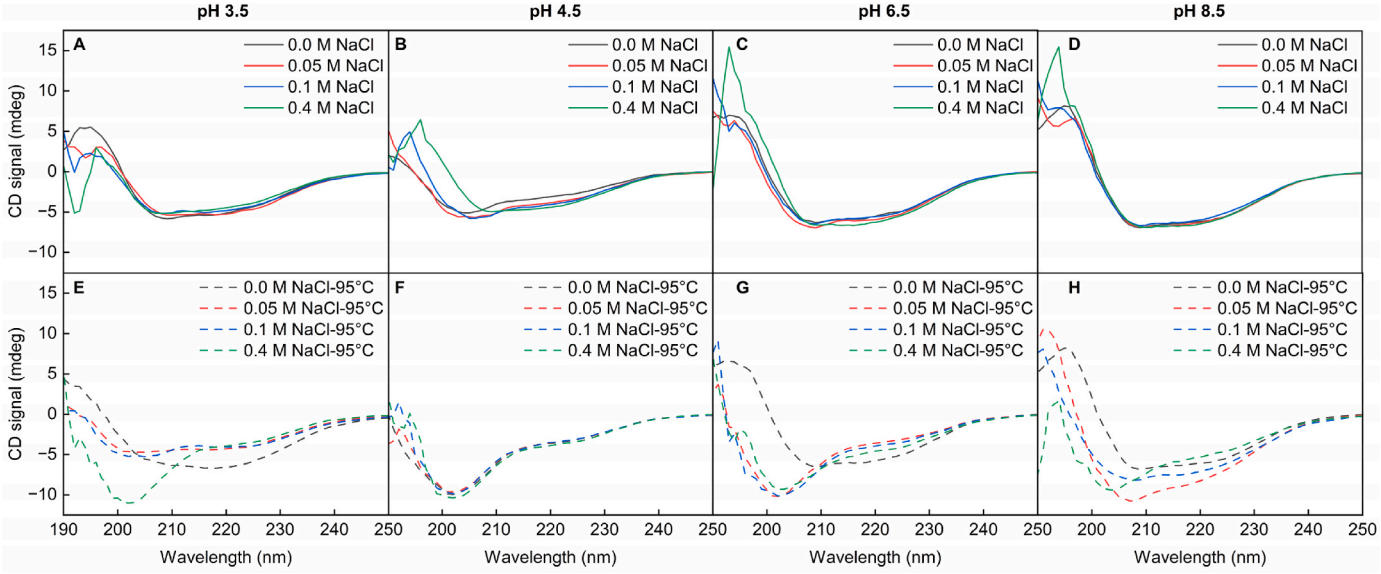

Fig. 5. Circular dichroism spectra of PPI resuspended at different pH (3.5 (A, E), 4.5 (B, F), 6.5 (C, G), and 8.5(D, H)) and at NaCl concentrations of 0 M (black), 0.05 M (red), 0.1 M (blue), and 0.4 M (green). Samples are supernatants diluted in 10 mM phosphate buffer, pH 7, to 0.1 mg/mL. Samples were compared before (solid lines, top) and after (dash lines, bottom) heat treatment (95 °C, 1 h).

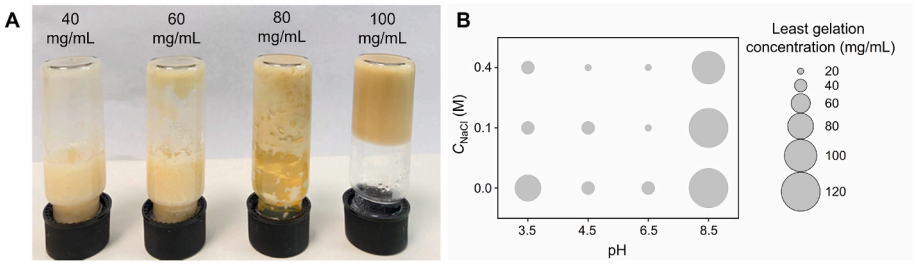

Fig. 6. (A) Effect of protein concentration on gelling of pea protein concentrate in water. (B) The least gelation concentration of PPI as a function of pH (3.5, 4.5, 6.5, and 8.5) in different salt solutions (0.0, 0.1, and 0.4 M NaCl).

increase of NaCl concentration to 0.4 M didn't change the LGC values. At high pH (pH 8.5), 0.4 M NaCl was required to reduce the LGC, although values were still high, compared to those at pH 4.5, where charges are minimized, and extensive aggregation occurs. This finding is consistent with prior studies, which suggest that the addition of salt can significantly lower the LGC (Guldiken et al., 2021). This effect may be attributed to the salt-induced extensive aggregation during heating, which promotes interactions between unfolded protein residues and enhances the formation of gel network.

3.7. Rheological properties of pea protein gels

Gels were prepared at their LGC and their viscoelastic properties evaluated using oscillatory rheology (Fig. 7). The G' dependence on frequency was analyzed by calculating the slope. In general, all the gels were behaving as physical gels regardless of pH and salt concentrations, in agreement with the literature, which describes these gels as formed by non-covalent interactions (Tanger et al., 2022).

Little frequency dependence indicates very elastic gel networks, formed with resilient molecular bridging, i.e. covalent bonds (Creusot et al., 2011), while higher frequency dependence (higher slopes) suggest more viscous particle gels. As shown in Fig. 7, the gels formed at pH 3.5 (Figs. 7A) and 4.5 (Fig. 7B) with different concentrations of NaCl displayed similar G' dependence with frequency, with a slope of 0.14–0.15. On the other hand, the gels formed at pH 6.5 (Fig. 7C), showed a decrease from 0.15 at 0.0 M NaCl to 0.12 at 0.4 M NaCl, indicating that

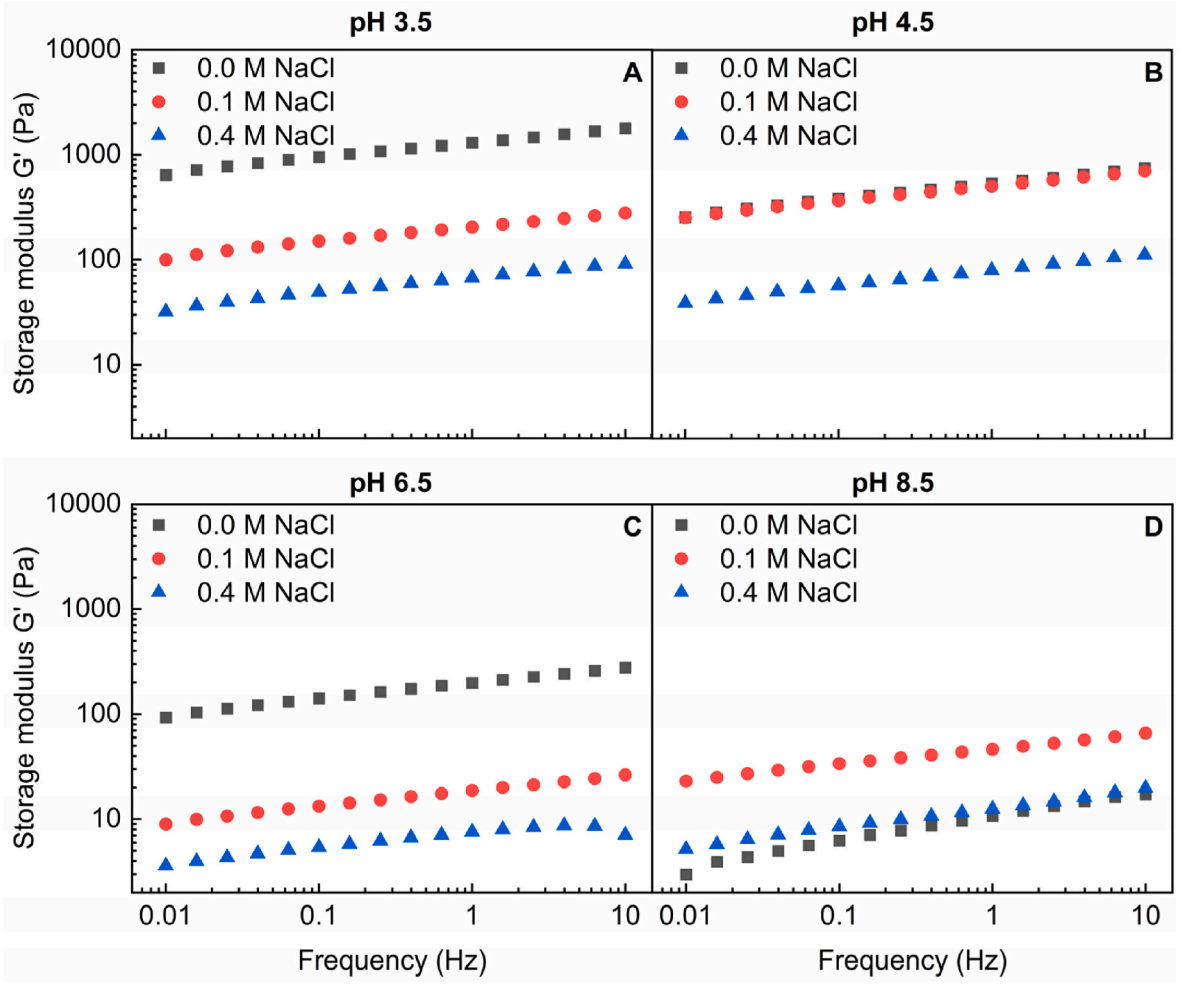

Fig. 7. Frequency sweep measurements of pea protein gels formed at the least gelation concentration for different solutions at different pH values of 3.5 (A), 4.5 (B), 6.5 (C), and 8.5 (D), containing 0.0 M (black squares), 0.1 M (red circles), and 0.4 M (blue triangles) NaCl.

salt addition influenced the gel network. Higher slope values were found in the gels formed at pH 8.5 (Fig. 7D), ranging from 0.15 (0.1 M) to 0.24 (0.0 M), suggesting a more viscous gel structure.

As protein concentration is an important factor in providing gel stiffness, Fig. 8 summarizes the storage modulus measured in all gels, at the corresponding LGC. It is clear that salt addition decreased gel stiffness at 1 Hz. The modulus was the highest at pH 3.5 (at an LGC of 80 mg/mL) and with no NaCl. Also gels at low ionic strength and pH 4.5 and 6.5 showed to be much stiffer than the gels containing NaCl. Furthermore, for the gels obtained at comparable LGC, a higher G' value was obtained for gels formed at pH 4.5 compared to pH 6.5. The gels formed at pH 8.5 exhibited the lowest gel stiffness even though made with the highest LGC (100–120 mg/mL), indicating the potential of modulating structure based on the characteristics of the intermediate protein aggregates.

For a better understanding of the structural differences of these gels in their linear viscoelastic range limit, Fig. 9 illustrates the critical stress and critical strain distribution of the gels obtained with different pH and ionic strength, at their respective LGC. Gels at low ionic strength exhibited higher critical strain values compared to their counterparts suspended in 0.4 M NaCl, except for those at pH 4.5, indicating that salt addition will result in brittle structures. The softest structures were obtained for gels containing 0.4 M NaCl.

Fig. 9 also shows that gels formed at the LGC at pH 3.5 in water were the toughest and most ductile gels, while the gels formed at LGC at pH 4.5 in water exhibited high critical stress but low critical strain, indicating a strong but brittle gel.

3.8. Heat-induced aggregation and gelation mechanism of PPI as a function of pH and salt

As illustrated in Fig. 10, the effects of NaCl on pea protein aggregation before heating are highly dependent on protein charge and pH conditions. Specifically, NaCl promoted aggregation in positively charged proteins (pH 3.5), while it enhanced the solubilization and dissociation of those proteins near their isoelectric point (pH 4.5, not shown due to the extensive precipitation) or at neutral pH (pH 6.5). At alkaline pH (pH 8.5), however, NaCl had a minimal impact on solubility due to the dominant influence of electrostatic repulsion.

During heating, the presence of salt facilitated the unfolding of PPI, exposing hydrophobic groups originally buried within the globulins. This exposure strengthened hydrophobic interactions between proteins,

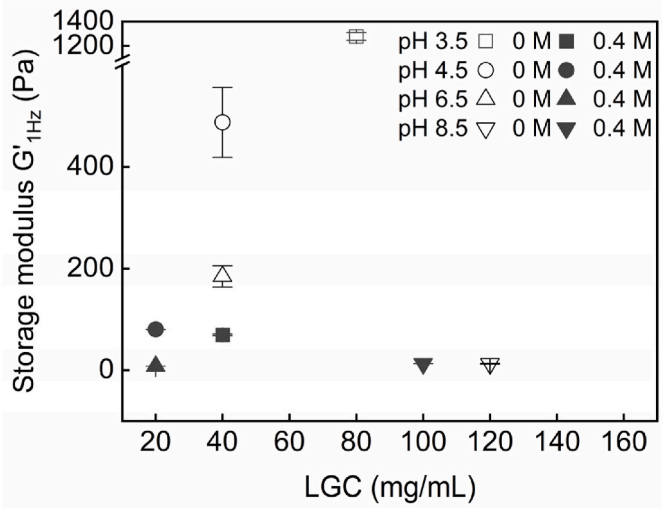

Fig. 8. Storage modulus (G') measured at 1 Hz for all protein gels formed at the least gelation concentration, plotted as a function of protein concentration, for different pH (3.5 (squares), 4.5 (circles), 6.5 (upright triangles), and 8.5 (inverted triangles) in 0.0 M (empty) and 0.4 M (solid) NaCl.

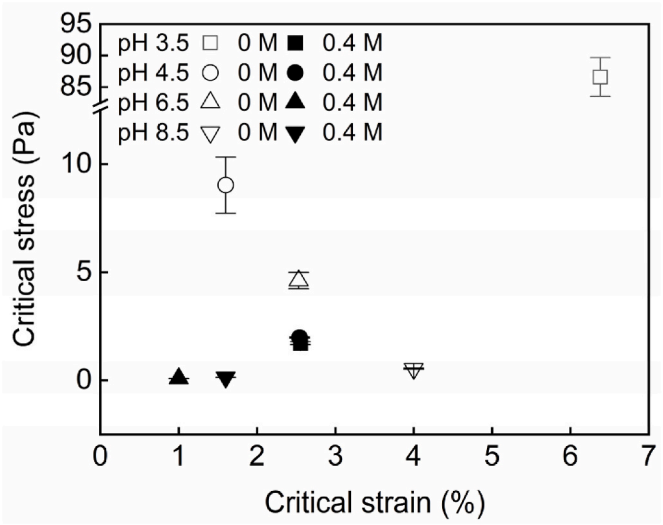

Fig. 9. Overview of the values of critical stress and critical strain γ_c measured from strain sweep measurements at the limit of the linear viscoelastic range of pea protein gels prepared at the least gelation concentration, at different pH (3.5 (squares), 4.5 (circles), 6.5 (upright triangles), and 8.5 (inverted triangles) in 0.0 M (empty) and 0.4 M (solid) NaCl.

contributing to the formation of a more robust gel network and reducing the least gelation concentration. However, excessive hydrophobic interactions also led to extensive protein aggregation, resulting in both soluble and insoluble aggregates. While aggregates improved gel stiffness, insoluble aggregates disrupted the gel structure, making salt-added gels more brittle and less stable compared to PPI gels formed in water without salt.

PPI gels formed at pH 3.5 without salt exhibited the toughest and most ductile structures, likely due to the lower formation of insoluble aggregates, which are detrimental to gel network integrity. In contrast, PPI gels formed at pH 6.5 with salt demonstrated higher stiffness but greater brittleness than those formed at pH 6.5 without salt. This behavior can be attributed to the higher proportion of aggregates, which acted as fillers within the gel network (Tanger et al., 2022). Notably, some larger insoluble aggregates disrupted the gel network, further compromising its stability. At pH 8.5, PPI gels displayed higher frequency dependence and lower gel stiffness, suggesting that they formed a viscous entangled fluid rather than a continuous gel network.

4. Conclusions

Salt extraction followed by dialysis can yield pea protein isolates (PPI) with high solubility and improved gelling capacity compared to pea protein concentrate. This PPI not only maintained its solubility in spite of the low ionic strength, but also showed high heat-stability. All the unheated pea protein samples had the lowest solubility at pH 4.5, apart from the solutions at 0.4 M NaCl, which contained more soluble globulins. All the unheated samples had the highest solubility at 0.4 M NaCl except at pH 3.5. Protein solubility of all samples in 0.4 M NaCl decreased after heating. The effect of salt on pea protein aggregation is distinct, depending on if the solutions are heated or nonheated, and it also varies with pH. This study, for the first time, elaborated the effect of salt on pea protein aggregation before and after heating, and the consequent effect on the gelation properties of pea protein. Before heating, salt presence decreased protein aggregation except for positively charged proteins. During the gelation process, salt addition, instead led to extensive protein aggregation. The aggregation facilitated gel network formation, which greatly reduced the least gelation concentration of pea protein gels. At the same time, extensive aggregation also produced a lot of insoluble aggregates, which are detrimental for gel

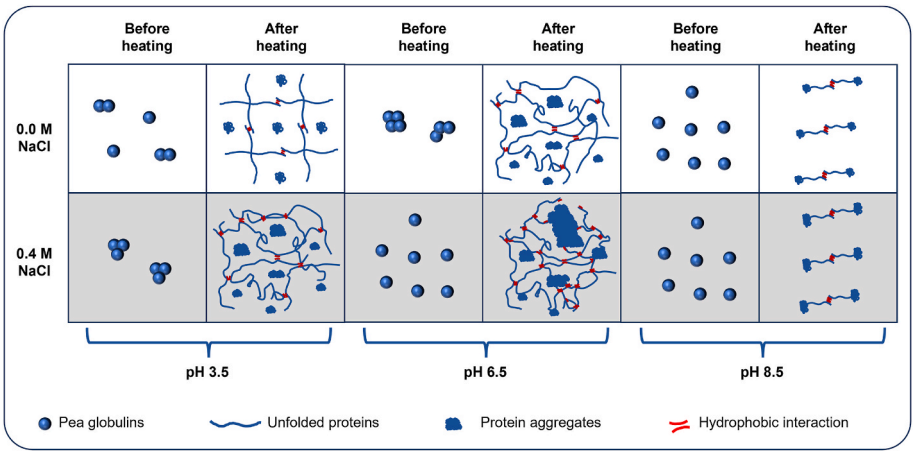

Fig. 10. Schematic aggregation and gelation process of salt extracted pea protein isolate under various pH and salt conditions.

network structure and stability, resulting in soft and brittle gels. These findings demonstrate the potential of a careful modulation of pH and NaCl concentration as an effective way to design gel structure in heat-set pea protein gels, by modifying the properties of the intermediate aggregates.

CRediT authorship contribution statement

Yi Zhang: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Anja Herneke:** Writing – review & editing, Supervision, Data curation. **Maud Langton:** Writing – review & editing, Supervision. **Mathias Johansson:** Writing – review & editing, Data curation. **Milena Corredig:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition.

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.

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Data availability

Data will be made available on request.

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