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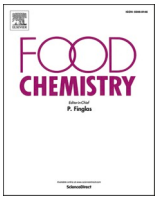

Effect of salt extraction on composition, structure, and thermal properties of pea protein

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ABSTRACT

Salt plays a vital role in modulating protein solubility during plant protein extraction. This study investigated the effect of salt concentrations during extraction on the composition, structure, and thermal properties of pea protein extracts. Low concentrations (0.0–0.2 M) of NaCl resulted in higher ratio of convicilin and vicilin with higher molar mass. Salt concentration did not affect the molar mass of legumin. With 0.4 M NaCl, protein extractability peaked at 78 % and extracted protein had the highest legumin-to-vicilin ratio. With NaCl concentrations greater than 0.4 M, protein composition of extracts remained unchanged, but the extractability decreased. Salt enhanced the heat stability of all pea proteins, as measured by NanoDSC. This study demonstrated that varying NaCl concentrations during protein extraction resulted in pea proteins with different compositions, structures, and thermal properties, offering valuable insights for developing customized protein extraction. The findings can also be extended to other plant proteins.

1. Introduction

In recent years, there has been an increased interest in plant-based proteins as a substitute for animal-derived proteins, driven by a variety of social, economic, and environmental factors (Kumar et al., 2022). Among the legumes, pea proteins are gaining particular attention due to their low allergenicity, non-GMO status, as well as their availability and low-cost production (Lam, Karaca, Tyler, & Nickerson, 2018). Pea seeds contain approximately 20–30 % storage protein, comprising water-soluble albumins (18–25 %), salt-soluble globulins (55–80 %), and minor amounts of glutelins and prolamins (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015).

Legume-derived storage proteins, depending on the source and processing history, exhibit a wide range of techno-functional properties, including solubility, emulsification, gelation, foaming, and water/oil retention capacity, which is of importance to their application in foods. A challenge to their adoption as ingredients in food is their substantial variability even within one plant origin, due to differences in processing history (Corredig, Young, & Dalsgaard, 2020). Dry fractionations utilize air classification to produce less-refined protein concentrates. This process preserves the proteins' native state, as the seed's protein bodies are still at least partly intact and have not been subjected to heat or other harsh treatments (Assatory, Vitelli, Rajabzadeh, & Legge, 2019; Schutyser, Pelgrom, van der Goot, & Boom, 2015). Aqueous isolations and subsequent fractionations are the most used methods to obtain protein ingredients, with aqueous extractions leading to higher purities (Dumoulin, Jacquet, Malumba, Richel, & Blecker, 2021). These methods include alkaline extraction-isoelectric precipitation (IEP), salt extraction-dialysis, and micellar precipitation (Stone et al., 2015). These methods take advantage of the different protein solubilities to pH or salt, to create ingredients of various composition. The isoelectric point of most proteins is in the pH range of 4 to 6, thus proteins at alkaline conditions are predominantly negatively charged and soluble (Novák & Havlíček, 2016). The presence of strong repulsive electrostatic forces between charged protein particles explains the higher protein extraction efficiencies obtained in alkaline environment. A widespread method to produce pea protein isolate is alkaline solubilization followed by isoelectric precipitation at pH 4.5. This treatment makes it possible to selectively precipitate the proteins that are close to their isoelectric point, including most globulins, while excluding those proteins and impurities remaining soluble in acidic environments. This process is quite efficient, resulting in high protein purities (Tanger, Engel, &

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Kulozik, 2020). However, recent research has proposed that during this process, the extreme local pH created during acidification induces some degree of irreversible aggregation of the globulins, leading to a ± 10 % decrease in protein solubility upon rehydration at pH 7 (Kornet et al., 2020; Yang & Sagis, 2021). In addition, the IEP protein products have been shown to be slightly colored due to co-extracted pigments, i.e., chlorophylls and polyphenols (Peng et al., 2024). The co-extraction of undesirable compounds, which could, for example, impart anti-nutritional properties, such as phytates, glucosinolates, tannic acid, and erucic acid, has also been reported (Kaspchak, Mafra, & Mafra, 2018; Peeters & Tenorio, 2022).

Salt addition can influence the interactions between protein molecules and phytic or tannic acid. Kaspchak et al. (2018) reported that high ionic strength increases the affinity between tannins and bovine serum albumin (BSA) and attributed this to the increased surface hydrophobicity of BSA at higher ionic strengths. On the other hand, high ionic strength decreases the interaction between phytic acid and BSA, by neutralizing the charged groups. Therefore, more attention should be given to the effect of salt in changing the population of co-extracted compounds during protein extraction. These non-protein components not only influence the protein purity in extracts but also often lead to undesirable sensory or antinutritional properties (Hadnađev et al., 2017).

Salt plays an important role during protein extraction mostly by modulating protein solubility, depending on the protein type. Below a critical salt concentration, increasing salt leads to salting-in effect (Gerzhova, Mondor, Benali, & Aider, 2016; Inyang & Iduh, 1996), while above this concentration salting-out effects occur (Bogahawaththa, Bao Chau, Trivedi, Dissanayake, & Vasiljevic, 2019; Lam et al., 2018). Salt extraction combined with dialysis has been suggested as a method to obtaining legumin-rich fractions. This method is based on the principle that proteins can be extracted in the presence of ions, but then can be precipitated when diluted to lower ionic strengths. This process can result in a mixture of globulins and albumins, with more native structures compared to isolates obtained from IEP (Stone et al., 2015; Tanger et al., 2020). Furthermore, ions present in the extraction buffer can exchange ions or shield surface charges by adsorbing to the colloidal structures' surfaces, causing changes in the electrical diffuse double layer (Hansen & Löwen, 2000), providing a more compact charged shell, increased charge screening, and reducing electrostatic repulsion between similar charged particles. This may aid in the extraction of protein from the carbohydrate-rich scaffold or may enhance the formation of protein aggregates, with a subsequent effect on the functionality of the ingredients obtained.

Recent studies have investigated the impact of salt on protein solubility and formation of aggregates during plant proteins extraction (Kornet et al., 2022; Schmitt et al., 2021; Tanger et al., 2020). The combination of varying salt concentrations and pH is also commonly used to selectively solubilize or precipitate legumin and vicilin fractions during protein fractionation (Chang et al., 2022; Rubio et al., 2014). Specifically, proteins are first extracted using borate buffer (pH 8) and 0.5 M NaCl. The pH is then lowered to 4.5 to precipitate legumins, while vicilins remain in the supernatant and are subsequently precipitated by salt removal through dialysis. Furthermore, more attention has been given to the effect of divalent ions in the formation of protein aggregates, as well as in the relationship between calcium ions and phytates in the extracts (Amat et al., 2024; Wang & Guo, 2021).

Hence, it is established that the presence of salt affects the solubility and the aggregation states of proteins (Jeganathan, Vasanthan, & Temelli, 2023). Therefore, a systematic study on the effect of salt concentration during extraction on the composition, structure, and colloidal properties of pea protein extracts is needed, as it will improve our understanding of the properties of pea proteins, and, ultimately, their techno-functionality.

The aim of this study was to investigate the impact of NaCl concentration during pea protein extraction on the composition, structure, solubility, and thermal properties of the suspensions. A dry fractionated pea protein concentrate derived from air classification was used as the initial extraction material, as in this concentrate, much of the seed architecture is still preserved (Dumoulin et al., 2021; Jeganathan et al., 2023). Insights from this study may be useful in understanding how to fine tune the extraction of pea proteins. In addition, a more in-depth knowledge of how ions impact the structural and functional alterations of different protein fractions during pea protein extraction can have broader applications for understanding other legume storage proteins.

2. Materials and methods

2.1. Materials

Pea protein concentrate (PPC) obtained from air classification, supplied by Vestkorn, located in Holstebro, Denmark, was utilized as the primary raw material for extraction process. According to the manufacturer, this concentrate contains 49 % protein on a dry weight basis. Additionally, the concentrate consists of 17 % total carbohydrates (including 4 % starch) and 16 % dietary fiber, 5 % fat, and 6 % ash, on dry basis. MilliQ water with a resistance of approximately 18.4 Ω was used throughout the entirety of the study. Sodium chloride (NaCl, ≥99.5 %) was purchased from VWR (USA). Sodium hydroxide (NaOH, ≥97 %), Sulfuric acid (H_2SO_4, ≥98 %), and Phenol (C_6H_6O, ≥99.5 %) were obtained from Sigma-Aldrich (USA). Hydrochloric acid (HCl, 37 %) was purchased from Fisher Scientific (USA). All other chemicals were of analytical grade and purchased from regular suppliers. All experiments were conducted at room temperature (22 °C) unless otherwise stated.

2.2. Samples preparation

Dry protein concentrates (10 g) were dispersed in a series of different concentrations of NaCl (0.0–1.2 M, 100 mL) to a final concentration of 10 % (*w/v*), with pH adjusted from 6.3 (0.0 M NaCl), 6.0 (0.2 M NaCl), 5.8 (0.4 M NaCl), 5.8 (0.6 M NaCl), 5.7 (0.8 M NaCl), 5.6 (1.0 M NaCl), 5.6 (1.2 M NaCl), to 7.0 using 1 M NaOH, and stirred on a magnetic stirrer for 2 h, while the pH was continuously monitored. The suspensions were then centrifuged (4500 *g*, 4 °C, 20 min) and supernatants were collected for further analyses and named depending on the concentration of NaCl (0.0–1.2 M) applied. NaN_3 (0.04 %, *w*/w, 0.006 M) was added to the extracted supernatants to inhibit bacterial growth. After extraction, samples were stored at 4 °C or freeze dried. Two independent extractions were carried out.

2.3. Composition of pea protein extracts

Total nitrogen content in PPC and protein extracts was determined by Dumas nitrogen analyzer (DUMATHERM®, Gerhardt Analytical Systems, Germany). Protein content was calculated using total nitrogen content to multiply with a protein conversion factor of 5.4 (Mariotti, Tomé, & Mirand, 2008), after adjusting for the NaN_3 added. Total carbohydrates were measured using Phenol-sulfuric acid method according to Nielsen (2010) with some modifications. In brief, the sample (0.2 mg) was dissolved in 1 mL MilliQ water, then mixed with 1 mL 5 % Phenol, followed by a hydrolysis by adding 5 mL concentrated sulfuric acid, and then stood at room temperature for 20 min. The absorbance of reacted sample solution was measured at 490 nm with a UV spectrophotometer (UV-3100PC, VWR, China) subtracted from a blank of 1 mL water without the sample. Glucose was used as a standard to build the calibration curve following the same procedure that was used for the sample.

Ions play a significant role in protein solubility, and some divalent cations, such Ca^{2+}, and Mg^{2+} can interact with proteins and/or other non-protein compositions, such as phytic acid, forming soluble or

insoluble complex (Amat, Assifaoui, Schmitt, & Saurel, 2023). The ionic composition was measured using Agilent 7900 inductively coupled plasma mass spectrometry (ICP-MS) equipped with a quadrupole MS detector (Agilent Technologies, US). First, samples and standard reference material (NIST SRM 3234) were weighed about 0.2 g and added to digestion quartz vessels and reacted with subsequently added concentrated HNO_3 (5 mL), 30 % H_2O_2 (2 mL), and concentrated HCl (1 mL) in fume hood overnight. Then, partially degraded samples were put in the XQ80–0 rotor of Multiwave 3000 microwave system (Anton Paar GmbH, Graz, Austria) for further digestion, using the following digestion program: power 1400 W, ramp 10 min, and hold for 15 min. ICP standard solutions of potassium (K^+) and calcium (Ca^{2+}), sodium (Na^+), magnesium (Mg^{2+}), and phosphorus (P) (Merck KGaA, Germany) were determined to establish a calibration curve (0–50 mg/L). Digested samples were ×5 diluted with MilliQ water prior injection. Argon was used as plasma gas and Helium was ORS (octopole reaction system) gas. The total phosphorus content was used as an indicator of phytates, due to the fact that phytic acid, also known as myoinositol hexaphosphate (IP6), along with its degraded forms of inositol phosphates (IP1 – IP5), constitute approximately 90 % of total phosphorus (P) in legumes (Helfrich & Bettmer, 2004).

2.4. Protein extractability

The protein extractability was measured by determining the amount of protein in the supernatant after centrifugation (4500 *g*, 4 °C, 20 min), and it was reported relative to the original protein content in PPC. Protein content in supernatant and PPC were determined using the Dumas method as mentioned above, after correction for the N contribution from NaN_3.

2.5. Particle size and surface charge (ζ-potential)

Protein extracts were diluted to 1 mg protein/mL using their corresponding extracting salt solution to see their aggregation state during extraction with various salt concentrations (0–1.2 M NaCl). The apparent hydrodynamic diameter was then measured using a Malvern Zetasizer Nano S (Malvern Panalytical Ltd., UK) with side scattering at a detection angle of 90°. Samples for ζ-potential experiments were diluted in phosphate buffer (1 mg protein/mL, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0) to ensure pH and a similar ionic environment, as this test is affected by the ionic environment.

2.6. Surface hydrophobicity (H_0)

The surface hydrophobicity index (H_0) of pea protein fractions was measured using the fluorescent probe 8-Anilinonaphthalene-1-sulfonic acid (ANS) according to previous reports (Haskard & Li-Chan, 1998) with some modifications. ANS (8 mM) was prepared in different concentrations of NaCl solutions (0.0 M – 1.2 M). Pea protein extracts were diluted to different concentrations of 0.02, 0.04, 0.06, 0.12, and 0.16 mg protein/mL, using the corresponding extraction NaCl solutions, to maintain their ionic environment. Aliquot (20 μL) of ANS was mixed with 4 mL of protein samples, reacted for 15 min in dark environment. The mixture was then loaded in a fluorescent cuvette and measured with a fluorescence spectrometer (FL 6500, PerkinElmer, US). The fluorescence intensity was measured at excitation and emission wavelengths of 390 nm and 470 nm using a 5 nm slit width. The background of NaCl solutions were deducted from the samples intensity results, and the background subtracted fluorescence intensities were plotted against corresponding protein concentrations. The slope from the plotted linear regression analysis was reported as hydrophobicity index H_0.

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

To evaluate differences in the polypeptide composition in the various extracts, the extracts were analyzed using non-reducing SDS-PAGE (Grasberger, Hammershøj, & Corredig, 2023). After extraction and centrifugation, the samples were mixed with MilliQ water and NuPAGE™ LDS sample buffer (4×) to the final loading protein concentration of 1 mg protein/mL. After heating (95 °C, 5 min) and centrifugation (4 °C, 10,000 *g*, 5 min) (Eppendorf microcentrifuge 5417R, Merck KGaA, Darmstadt, Germany), the prepared samples (10 μL) and pre-stained protein ladder standard (5 μL) (Spectra™ Multicolor Broad Range Protein Ladder) were loaded to the NuPAGE™ (4–12 %, gradient) Bis-Tris precast mini protein gels fixed in the Invitrogen™ XCell SureLock™ Mini-Cell. The electrophoresis was run under 200 V for 35 min after filled with NuPAGE™ MES SDS running buffer. After fixing with a solution containing 50 % ethanol, 8 % phosphoric acid for 2 h, the gel was stained 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, followed by destaining by immersing in water for 1.5 h. The gel was imaged using the Gel Doc™ EZ System (Image Lab™, BIO-RAD laboratories, USA) and analyzed with Image Lab software (Bio-Rad lab., US).

2.8. Size-exclusion chromatography coupled with multi-angle-light-scattering and refractive-index detectors (SEC-MALS-RI)

The protein composition of the various extracts, as well as their molar mass distribution was studied by SEC-MALS-RI. In this case, to ensure a stable pH and sufficiently high ionic strength during elution, the extracted pea protein dispersions were mixed with phosphate buffer (38 mM Na_2HPO_4, 12 mM NaH_2PO_4, 150 mM NaCl, 0.02 wt% NaN_3) to a final protein concentration of 6 mg/mL. Bovine serum albumin (BSA) was used for normalization, inter-detector alignment correction, and band broadening, and it was dissolved in the same running buffer to 2 mg/mL. All samples were filtered with a 0.45 μm PVDF membrane before injection (20 μL). The high-performance liquid chromatography (HPLC) system (Agilent Technologies 1260 Infinity series, California, US) was equipped with a Yarra SEC-4000 column (300 × 7.8 mm), particle size 3 μm, connected with a GFC-4000 (4 × 3.0 mm internal diameter) SecurityGuard Cartridge (Phenomenex, US). The HPLC system was coupled to 3 detectors: a 1260 Infinity II Diode Array Detector (Agilent Technologies, California, US) to determine protein concentration via absorbance at a wavelength of 280 nm, an 18-angle DAWN Heleos II multiangle laser light scattering (MALS) detector to measure the scattered light intensity from samples into multiple angles relative to the incident laser beam, and a Shodex RI-501 refractive index (RI) detector (Showa Denko K. K., Japan), which also measures protein concentration based on the refractive index change caused by the proteins in solution. The refractive index increment (dn/dc) for proteins in the phosphate buffer was set as 0.185 mL/g. ASTRA 8.1.2 software (Wyatt, Santa Barbara, California, US) was used for data acquisition and data analysis. The relative proportion of different protein compositions of main globulins was calculated using the peak area relative to the total peak area of all the main globulins. The M_w was calculated using a Zimm plot model (ASTRA, Wyatt) according to the first principle in Eqs. (1) and (2) (Wyatt, 1993),

$$K^* = 4\pi^2(dn/dc)^2 n_0^2 \Big/ \left(N_a \lambda_0^4\right) \quad (1)$$

where K^* is an optical constant, dn/dc is the refractive index increment of the solution, n_0 is the refractive index of the solvent, N_a is Avogadro's number, and λ_0 is the vacuum wavelength of the incident light.

$$K^* c/R(\theta, c) = 1/M_w P(\theta) + 2A_2 c \quad (2)$$

where θ is the angle between the incident light and the scattered light, c is the analyte concentration, M_w is molar mass of the analyte, $R(\theta, c)$ is the excess Rayleigh ratio of the solution as a function of scattering angle θ and concentration c, A_2 is the second virial coefficient, and $P(\theta)$ refers to the angular dependence of the scattered light.

2.9. Fluorescence measurements

Intrinsic fluorescence was measured in a fluorescence cell (ES Quartz Glass, Aireka Scientific Co., Ltd., Hongkong, China) using a LS 50B spectrofluorometer (Perkin-Elmer, Beaconsfield, England) with a selective excitation of tryptophan at 298 nm and maximum emission record from 305 to 450 nm at a resolution of 0.5 nm. All samples were diluted with phosphate buffer (5 mM Na_2HPO_4, 5 mM NaH_2PO_4, pH 7.0) to 0.1 mg protein/mL, to maintain a similar ionic environment, while analyzing their structure. Data was obtained after subtraction of phosphate buffer background.

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

Small-angle X-ray scattering intensities of extracted pea protein fractions and corresponding backgrounds were measured using a Xenocs Nanoinxider (Grenoble, France) with a Cu-Kα source and covering a q range of 0.004–0.3 $Å^{-1}$. All pea protein fractions were suspended to a protein concentration of 4 mg/mL in corresponding concentration of NaCl solution, as their aggregation state may be affected by the ionic environment. The background corresponding to each solution was prepared by using centrifugal filters (cut off 10 kDa, Pall Corporation). The suspension was centrifuged at 4500 g for 20 min with corresponding concentration of NaCl solution and then the liquid filtered through the membrane (permeate) was used as the background. The XSACT software was used for log-binning and background subtraction. Potential differences in the scattering curves were estimated using a unified fit equation which uses a power law combined with Guinier plot followed by a power law, using the Irena package (Beaucage, 1995; Ilavsky & Jemian, 2009).

2.11. Thermal analysis

Thermal properties of the samples were analyzed using Nano-Differential Scanning Calorimetry (NanoDSC) (TA instruments, US). Pea protein fractions extracted with NaCl solutions with different concentrations (0.0–1.2 M) were loaded directly to the sample cell with the respective NaCl solutions in the reference cell, without dilution, and then heated from 25 °C to 120 °C at the heating rate of 1 °C/min at 5 bar. All samples were degassed before analysis. The denaturation temperature T_d and enthalpy change ΔH were determined as the peak temperature and the area under the heat flow curve, respectively, and the enthalpy was normalized to J/g protein, using the software (NanoAnalyze, TA Instruments, US) provided by the manufacturer.

2.12. Statistical analysis

All extractions were conducted as two independent experiments and analyses were performed at least with two technical replicates, and results were expressed as mean value ± standard error ($n = 2$).

3. Results and discussion

3.1. Composition of the salt extracted fractions

The different salt concentrations used during pea protein extraction will result in the co-extraction of other components, which can form complexes. This is important as some of these components (i.e. soluble polysaccharides, oligosaccharides, phytates and organic acids) may cause changes not only to the protein solubility but also other functional properties. The composition of the various freeze-dried extracts is summarized in Table 1. The original pea protein concentrate, PPC, contained 5 % ash. The ions present, as measured by inductively coupled plasma-mass spectrometry, were 0.0 % Na^+, 1.5 % K^+, 0.1 % Ca^{2+}, 0.2 % Mg^{2+}. When extracted with 0 M NaCl, the extract exhibited higher ash content, primarily due to the enrichment of soluble fractions and the limited solubility of the globulins. The addition of NaN_3 also contributed to the higher ash content in the 0.0 M NaCl extract. Furthermore, the slightly higher protein content in the 0.0 M NaCl extract compared to the PPC can be partially attributed to the NaN_3 addition. However, the primary reason for the increased protein content after aqueous extraction is the removal of insoluble components during centrifugation. It is important to note that the influence of NaN_3 addition is negligible when comparing different extracts, as the same amount was added to all protein extracts. As expected, the ash content in the freeze-dried extract increased with higher NaCl concentrations during aqueous extraction, likely due to the incremental salt addition (~0.6 g/100 mL per 0.1 M NaCl) during the extraction process. This will exert a huge influence on the sensory properties of protein products; thus, the salt removal step is necessary for subsequent food application. However, in this study, although the increasing ash content exerted a huge influence on the content of other compositions, we didn't remove salt to avoid the composition change during dialysis. Instead, we compared the relative ratio of different compositions to see their compositional change as an effect of NaCl concentration.

Carbohydrates in pea mainly consist of starch, dietary fiber, and non-starch carbohydrates such as sucrose, oligosaccharides, and cellulose (Lam et al., 2018). As shown in Table 1, the PPC retained a high carbohydrate residue (49 %) despite starch removal through dry fractionation. This value is much higher than the total carbohydrate content reported by the manufacturer (33 %). The discrepancy may be attributed to differences in measurement methods. The manufacturer likely used the difference method, estimating carbohydrate content based on the levels of other components. In contrast, this study employed the phenol-sulfuric acid method, a widely used colorimetric approach for direct quantification of total carbohydrates. During the aqueous extraction, some of the starch (insoluble starch) and the insoluble fiber were removed, and their possible interaction with proteins will influence the protein extraction efficiency. The existence of carbohydrates not only result in lower protein purity of the protein extract, but also may further influence the techno-functional properties of pea protein products (Sim & Moraru, 2020; Yang, Zamani, Liang, & Chen, 2021). With increasing NaCl in the extraction buffer, the ratio between protein and carbohydrates in the freeze-dried extracts increased from about 1 at low salt concentrations, to above 2 with 1.2 M NaCl extraction. This change in composition as a function of NaCl clearly demonstrated that the presence of salt improved the dissociation of the protein from the complex architecture of the protein bodies, thereby improving protein extractability. This could be attributed to the salt screening effect on electrostatic interactions, potentially disrupting salt bridges between charged groups of proteins and carbohydrates. As a result, salt destabilizes their interactions and enhances protein extraction efficiency.

To better understand the ions distribution in the extracts, the amount of K^+, Ca^{2+}, Mg^{2+}, and total phosphorous (P) was measured in the freeze-dried samples (Table S1). Due to the high ash content of the extracts, the values of individual ions are also reported relative to the protein content in the various extracts in Table 1. Notably, the increase of phosphorous amount in the extracts indicates co-extraction of phytates with the proteins. This observation aligns with a recent study, which not only reported the formation of binary complexes between pea protein and phytic acid but also highlighted that their interactions vary depending on the pH environment (Amat et al., 2024).

The ratio of phosphorous/protein was higher in albumin rich extracts (low salt extracts) compared to the extracts rich in globulins, at NaCl concentrations ≥0.4 M, consistent with prior findings (Lombardi-Boccia, Carbonaro, Lullo, & Carnovale, 1994) for studies on white beans,

Table 1
Composition of freeze-dried salt extracted pea protein fractions (0.0 M–1.2 M NaCl), and of the original pea protein concentrate (PPC). Values are shown as mean ± standard error ($n = 2$).

Samples	Protein (%)	Total Carbohydrates (%)	Protein / Total carbohydrates	Ash (%)	K^+ (mg/g protein)	Ca^{2+} (mg/g protein)	Mg^{2+} (mg/g protein)	P (mg/g protein)
PPC	45.7 ± 0.0	48.8 ± 1.4	0.94 ± 0.02	5.1 ± 0.1	33.2 ± 0.8	1.85 ± 0.04	4.34 ± 0.03	12.6 ± 0.1
0.0 M NaCl	47.9 ± 0.0	46.6 ± 1.3	1.03 ± 0.02	11.5 ± 0.0	84.0 ± 3.1	2.97 ± 0.18	10.40 ± 0.77	28.9 ± 0.4
0.2 M NaCl	40.6 ± 0.0	27.6 ± 2.2	1.48 ± 0.08	24.6 ± 0.1	79.5 ± 1.6	2.87 ± 0.01	9.62 ± 0.58	26.0 ± 0.3
0.4 M NaCl	40.9 ± 0.1	25.3 ± 2.8	1.62 ± 0.13	32.2 ± 0.1	62.4 ± 2.8	2.49 ± 0.11	7.63 ± 0.58	21.7 ± 0.7
0.6 M NaCl	36.0 ± 0.1	18.3 ± 0.2	1.97 ± 0.01	40.2 ± 0.1	62.6 ± 3.5	2.57 ± 0.13	7.66 ± 0.65	22.0 ± 1.0
1.2 M NaCl	27.7 ± 0.1	12.7 ± 2.3	2.21 ± 0.29	54.3 ± 0.1	63.4 ± 3.5	2.83 ± 0.39	7.69 ± 0.74	21.7 ± 0.4

indicating a possible interaction between phytic acid and pea globulins. The most abundant cations (except for Na^+) present in the extracts were K^+ and Mg^{2+}, with levels decreasing with NaCl ≥0.4 M. This decline followed a similar trend to the relative phosphorus content, suggesting the possible formation of binary complexes between cations and phytic acid, as well as potential ternary complexes involving pea globulins, phytic acid, and cations. Amat et al. (2024) reported the formation of insoluble binary protein-Ca^{2+} complex at pH 6.5 and insoluble ternary complexes among pea protein, Ca^{2+}, and phytic acid at pH 7, which could also reduce protein solubility and lead to lower protein extractability.

3.2. Protein extractability as a function of NaCl concentration

Protein extractability, which is the amount of protein extracted in the supernatant after centrifugation relative to the protein content in the original sample, was measured as a function of NaCl concentration. Table 2 shows the protein extractability, zeta potential, and surface hydrophobicity of pea protein concentrates extracts at different salt concentrations. The protein extractability increased from 49 % of the total protein in the concentrate with no NaCl added and reached a maximum of 78 % in the presence of 0.4 M NaCl. Considering that the main storage proteins in pea are salt-soluble globulins (Hurkman & Beevers, 1980), the increase in protein extractability at 0.4 M NaCl can be partly attributed to the salting-in effect (Boire et al., 2019), but also to monovalent ions displacing salt bridges between proteins and proteins-cell wall polysaccharide chains (Uruakpa, 2012; Warnakulasuriya & Nickerson, 2018). A similar effect on protein extractability as a function of NaCl concentration has also been reported with other legumes, such as faba bean (Jeganathan et al., 2023), chickpea (Osman, Hassan, Ali, & Babiker, 2005), cowpea, and pigeon pea (Ahmed et al., 2012); most cases showed a maximum extractability at about 0.4 M NaCl, with a decrease at higher salt concentrations. At NaCl concentrations exceeding 0.6 M, extractability decreased, likely due to the salting-out effect.

Table 2
Protein extractability, zeta potential, and surface hydrophobicity of different pea protein fractions extracted with 0.0–1.2 M NaCl. Protein extractability indicates the proportion of soluble protein recovered in the supernatants (% of total protein in the original concentrate) after extraction with varying concentrations of NaCl. Samples for zeta potential were diluted to 1 mg protein/mL with phosphate buffer (5 mM, pH 7.0). Values are shown as mean ± standard error (n = 2).

Samples	Protein extractability (%)	Zeta potential (mV)	Surface hydrophobicity (H_0*10^{-3})
0.0 M NaCl	48.7 ± 0.8	−21.5 ± 0.2	57.6 ± 2.6
0.2 M NaCl	57.7 ± 0.2	−17.6 ± 1.5	62.4 ± 4.3
0.4 M NaCl	77.8 ± 2.3	−17.3 ± 0.2	89.3 ± 2.4
0.6 M NaCl	77.6 ± 0.3	−16.7 ± 0.0	85.8 ± 1.2
1.2 M NaCl	69.0 ± 0.3	−13.1 ± 0.3	136.3 ± 8.3

Hence, only samples extracted with NaCl concentrations ranging from 0.0 to 0.6 M, and the highest concentration of 1.2 M were chosen for further experiments.

The ζ-potential of the protein suspensions was the highest (−21.5 ± 0.2 mV) at 0 M NaCl and reached a lower plateau at about −17 mV at 0.2–0.6 M NaCl, with a lower value at the highest salt concentration, −13.1 ± 0.3 mV at 1.2 M NaCl. A similar decrease of ζ-potential with increasing NaCl concentration has been reported also for soy protein suspensions (Tian et al., 2021; Zhang, Lin, Zhang, & Tang, 2022). The decrease in ζ-potential with increasing NaCl concentrations indicates that salt modified the overall surface charge of proteins, reducing electrostatic repulsion. This, in turn, lowered system stability, leading to enhanced aggregation and decreased protein extractability. In addition, the increased surface hydrophobicity for the extracts extracted with increasing NaCl (0.0–1.2 M) indicated the exposure of hydrophobic residues from protein, which could be caused by dehydration by high concentrations of strongly hydrated cations (Zhang, Jeganathan, Dong, Chen, & Vasanthan, 2021). Consequently, the rise in surface hydrophobicity with higher concentrations of NaCl favored protein-protein interactions and aggregation, further explained the decreased protein extractability at NaCl concentration higher than 0.6 M.

3.3. Protein composition

3.3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of various extracts

SDS-PAGE was performed to evaluate possible differences in polypeptides' composition after extraction with different concentrations of NaCl. Globulins and albumins are the main proteins in peas, accounting for 55–65 % and 18–25 %, respectively (Lu, He, Zhang, & Bing, 2020). The major components in globulins are legumin, convicilin, and vicilin (Dziuba, Szerszunowicz, Nałęcz, & Dziuba, 2014). Legumin is a hexamer with a molecular weight of 350–380 kDa, and its monomer is composed of an acidic subunit (40 kDa) and a basic subunit (20 kDa), normally linked through disulfide bonds (Lu et al., 2020). Vicilin is a trimer quaternary structure with a molecular weight of 150 kDa, composed of monomers with molecular weight around 48–50 kDa (Lu et al., 2020). The light band migrating at about 94 kDa can be attributed to lipoxygenase (LOX) which is also present in pea protein seed. The band at 71 kDa band was recognized as convicilin, which can form trimers of around 210 kDa (Emkani, Oliete, & Saurel, 2021). Vicilin fragments α, β, and γ, with a molecular weight of 19 kDa, 13.5 kDa, and 16 kDa, respectively, were also present in all salt extracts, as well as albumins (10 kDa) (Dziuba et al., 2014).

Fig. 1A illustrates the difference in polypeptide composition in extracts obtained with varying NaCl concentrations, loaded with equivalent protein concentrations, under non-reducing condition. There were clear changes in the intensity of specific protein bands. As expected, due to its salt-dependent solubility, the legumin bands intensities increased from 0.0 M to 0.4 M NaCl and exhibited no further changes at higher NaCl concentrations (> 0.4 M). Furthermore, the convicilin band intensity increased from 0.0 M to 0.2 M NaCl and remained stable thereafter. Less differences were observed for the vicilin monomer, as well as for the acidic and basic fractions of legumin, not crosslinked, also

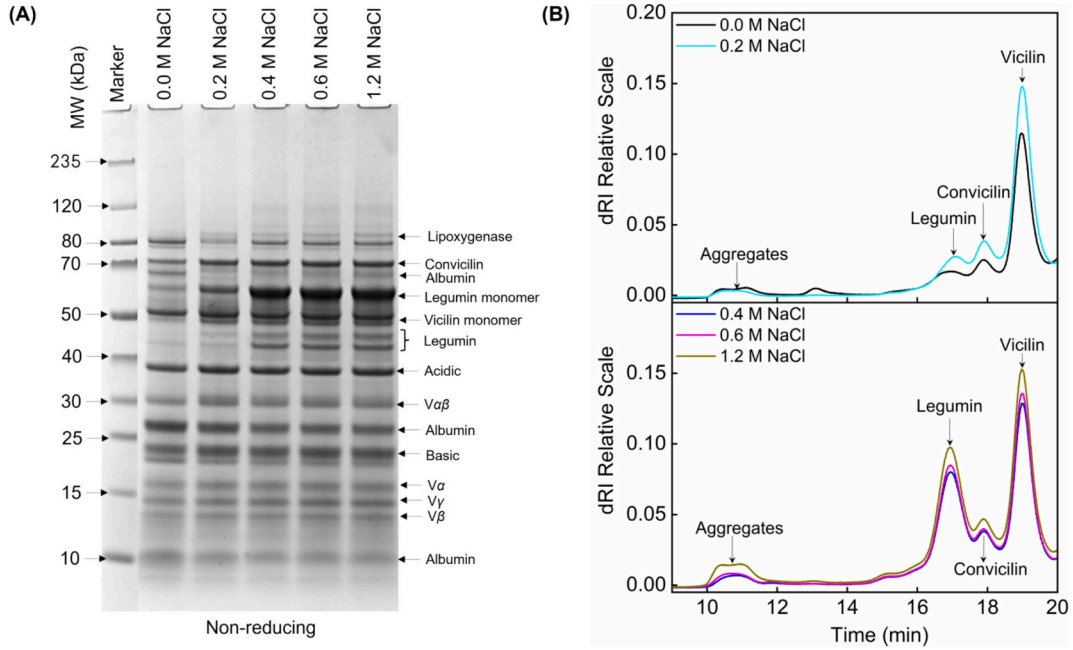

Fig. 1. (A) SDS-PAGE profiles of pea protein extracted with different concentrations of NaCl solutions under non-reducing condition. Proteins were loaded at a concentration of 1 mg protein/mL. Acidic is the acid subunit of legumin and Basic is the basic subunit of legumin. V_{α}, V_{β}, V_{γ}, and $V_{\alpha\beta}$ are the different subunits of vicilin. (B) Size exclusion chromatograms (dRI relative scale) as a function of retention time for the soluble fraction of pea protein extracted with different concentrations of NaCl (0.0–0.2 M, top; 0.4–1.2 M, bottom).

present in the extracts, regardless of the salt concentration. Distinct acidic and basic subunits of legumin in pea protein under non-reducing condition were also found in other research work (Grasberger et al., 2024; Peng et al., 2016), suggesting that some of these subunits are also present in the seed non-crosslinked in the legumin. In contrast, the band intensity of vicilin subunits $V_{\alpha\beta}$ decreased with extracts containing more than 0.2 M NaCl. Additionally, in the fractions extracted with high salt concentrations, the intensities of albumin bands around 11.5, 26, and 68 kDa decreased, with the latter even disappearing (Maningat, Jeradechachai, & Buttshaw, 2022; Yang et al., 2021), consistent with the fact that legumin is a salt-soluble globulin, whereas albumin is water-soluble (Lu et al., 2020). In conclusion, it was clear that in extracts containing less than 0.4 M NaCl (about 2.3 % salt), the composition of protein was strongly dependent on salt concentration. Despite the differences in protein extractability shown in Table 2, the ratios of legumins to vicilin, convicilin, and albumins did not seem to further vary at NaCl concentrations >0.4 M.

3.3.2. *Size-exclusion chromatography coupled with multi-angle light scattering and refractive-index detectors (SEC-MALS-RI)*

To better evaluate the relative proportion between the proteins, size exclusion chromatography was combined with multi-angle light scattering (SEC-MALS) to evaluate the concentration and molar mass of protein fractions in the extracts. Fig. 1B shows the elution profile of various extracts obtained by size exclusion chromatography. In addition to a small aggregate peak eluting at 10 min, all fractions displayed three distinct peaks attributed to legumin, convicilin, and vicilin proteins. The elution profiles were generally divided into low salt group (0.0–0.2 M NaCl) and high salt group (0.4–1.2 M NaCl) according to elution chromatographic similarity. The peak areas under those peaks were evaluated as an indication of the relative proportions of different fractions, as shown in Table 3. Soluble aggregates exhibited the lowest ratio to total area in the extracts at 0.2 M NaCl. In all other cases, the aggregates area was statistically equivalent. The legumin peak reached its maximum at 0.4 M NaCl, with about 38 % of legumin proteins, and was lower at the lower NaCl concentration, in agreement with the SDS-PAGE observations. The convicilin peak on the other hand, showed its highest ratio at 0.2 M, and then remained similar at higher NaCl levels. The vicilin relative area was the highest at low NaCl concentrations (0.0 M – 0.2 M), and then remained constant at higher NaCl.

3.4. *Colloidal state of extracted pea protein*

Various extraction conditions could also lead to different structure changes in different proteins (legumin, convicilin, and vicilin). Combining size exclusion chromatography and multi-angle light scattering makes it possible to evaluate molar mass changes of different protein fractions as an effect of NaCl concentration. A summary of the molar mass calculated for various fractions is also shown in Table 3. Notably, legumin in all samples showed a similar molar mass ranging between 293 and 322 kDa, regardless of NaCl concentration. In the case of convicilin and vicilin samples, the highest molar masses (175 and 145

Table 3
Relative proportion (based on area %) and molar mass of different protein fractions present in the various extracted pea proteins, and the legumin-to-vicilin ratio.

Samples	Relative ratio (%)				Legumin-to-vicilin ratio	Molar mass (kDa)		
	Aggregates	Legumin	Convicilin	Vicilin		Legumin	Convicilin	Vicilin
0.0 M NaCl	4.5 ± 0.5	16.7 ± 0.8	14.8 ± 0.3	63.9 ± 0.0	0.26	322 ± 21	179 ± 15	145 ± 2
0.2 M NaCl	1.5 ± 0.4	17.1 ± 0.1	16.1 ± 0.1	65.3 ± 0.4	0.26	306 ± 20	175 ± 3	146 ± 3
0.4 M NaCl	4.3 ± 2	38.0 ± 0.6	12.2 ± 0.2	45.5 ± 1.5	0.84	310 ± 2	126 ± 6	135 ± 5
0.6 M NaCl	4.4 ± 0.3	36.7 ± 0.0	12.5 ± 0.1	46.3 ± 0.1	0.79	308 ± 2	124 ± 6	137 ± 2
1.2 M NaCl	7.1 ± 1.3	36.4 ± 0.2	13.0 ± 0.3	43.4 ± 0.6	0.84	293 ± 1	112 ± 19	128 ± 4

kDa, respectively) were measured for the extracts at low salt concentration (0.0–0.2 M NaCl). At 0.4 M NaCl, convicilin and vicilin showed smaller molar masses (126 and 135 kDa, respectively) compared to those in the fractions extracted with low NaCl concentrations. This reduction in molar mass may be attributed to the salt-induced dissociation of non-covalently linked monomers or subunits within convicilin and vicilin (Pedrosa & Ferreira, 1994). With increasing salt (> 0.4 M NaCl), the molar mass of convicilin and vicilin remained unchanged. The lower sensitivity of legumin than convicilin and vicilin in the structure change with NaCl concentration could be due to the more compact structure of legumin. This phenomenon has also been observed by O'Kane, Happe, Vereijken, Gruppen, and van Boekel (2004), who found that vicilin and convicilin eluted out earlier at lower salt concentrations than legumin during chromatography fractionation using a salt gradient in the running buffer.

The apparent hydrodynamic diameter (D_h) of the protein suspensions was also measured using dynamic light scattering (Fig. S1). All extracts showed an intensity distribution showing predominantly submicron size aggregates. The apparent diameter was lower in the extracts between 0.0 and 0.6 M (185.9 ± 0.6 nm at 0.4 M NaCl, 185 ± 10 nm at 0.6 M), and larger, with larger uncertainties measured in the fractions extracted with higher amounts of salt (218 ± 17 nm at 1.2 M NaCl). At 1.2 M NaCl, the intensity distribution showed a shift in population size towards larger particles, likely due to enhanced aggregation resulting from reduced electrostatic repulsion and increased hydrophobic interactions. Meanwhile, a smaller particle population (~15 nm) also emerged in 1.2 M NaCl extract, which may correspond to an increased presence of globulins with molecular weights of 45–48 kDa, as indicated by SDS-PAGE results. Similar findings have been reported in the literature (Sarigiannidou et al., 2022) for pea protein hydrolysates, where the hydrodynamic diameter initially decreased up to 0.2 M NaCl and then increased with higher salt concentrations. DLS observation confirmed the presence of very large aggregates in the protein extracts, and the sensitive scattering of DLS to large particles may lead to inaccurate measurements of smaller and native proteins (Fischer & Schmidt, 2016). This brings up the importance of studying these systems at multiple length scales simultaneously.

3.5. Structural characterization

3.5.1. Intrinsic fluorescence

Protein intrinsic fluorescence, which primarily originates from the emission of aromatic amino acid residues, including phenylalanine, tyrosine, and tryptophan. The emission of tryptophan was used to study the tertiary structure change of pea protein extracts as a function of salt extraction (Fig. 2). All the extracted pea proteins displayed the maximum fluorescence emission intensity at the same wavelength around 340 nm (Table S2). There were differences in the fluorescence intensity, which can be attributed to differences in composition and aggregation state, as the proteins were all analyzed at the same concentration. Previous authors have reported that legumin of pea protein exhibited a higher fluorescence intensity than vicilin and albumin (Shen et al., 2023). However, in this work, the extract at 0.0 M NaCl showed the highest fluorescence intensity, even though it had the lowest level of legumin, and highest albumin and vicilin ratio compared to the other salt extracts. Although little compositional differences were noted in the legumin, convicilin, and vicilin fractions between the 0.0 M and 0.2 M extracts (Table 3), there was a marked decrease in fluorescence intensity. Possible explanations for the sharp decrease in fluorescence intensity could be the conformational change caused by the increased NaCl concentrations. At higher salt concentrations between 0.4 and 0.6 M, there was a similar ratio of legumin/vicilin, and similar molar mass of different protein fractions, indicating a similar aggregation state, therefore, their fluorescence intensities were comparable. Then the fluorescence intensity increased further at 1.2 M, due to the salt-induced dehydration of the protein surface. A similar trend, with a decrease in fluorescence intensity at low NaCl concentrations and increased at higher salt concentrations has also been reported in reconstituted pea protein isolate (Zhang et al., 2021).

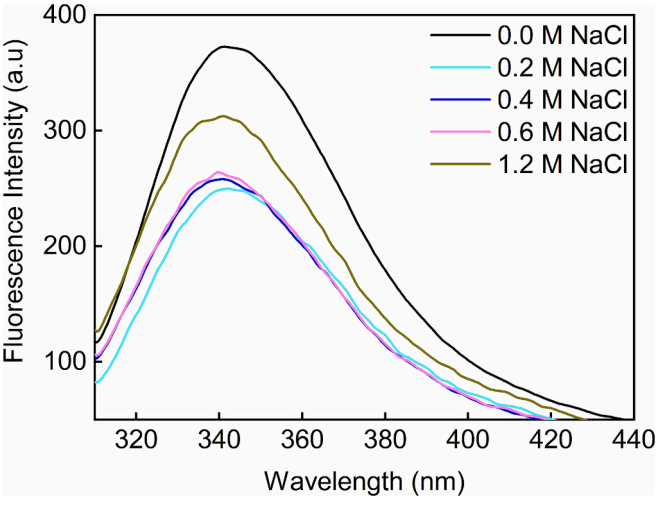

Fig. 2. Fluorescence spectra of pea protein fractions extracted with different NaCl concentrations, diluted with phosphate buffer (5 mM, pH 7.0).

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

Small-angle X-ray scattering (SAXS) allows to evaluate an ensemble average of conformational transitions and colloidal structures at multiple length scales, and, therefore, useful in understanding potential changes in the structural arrangements of the protein suspensions extracted at different salt concentrations. Fig. 3 shows the scattering intensity of all protein samples, at the same protein concentration, as a function of q. The q range used in this study probed two scattering features for all salt-extracted samples. First, the shoulder around 0.05 $Å^{-1}$, also called Guinier regime, was visible in all samples, apart from the 0.0 M NaCl extracted sample, which had a bend shifted to lower q values compared to the salt extracts, indicating the presence of larger local structures at low ionic strength. This area has been recently reported also for pea protein isolates prepared by isoelectric precipitation (Chen, Kuzmenko, Ilavsky, Pinho, & Campanella, 2022). Furthermore,

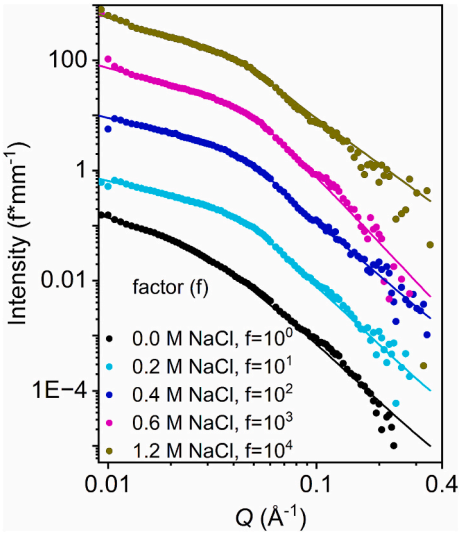

Fig. 3. SAXS scattering intensity profiles and unified fit (lines) of pea protein fractions extracted with different NaCl concentrations (0–1.2 M). The scattering intensity values of 0.0–1.2 M NaCl samples were multiplied with a factor (f) of 10^0, 10^1, 10^2, 10^3, and 10^4 to separate the curves for comparison.

at the lower q values, an up-turn slope was noted in all samples, with the water extract (0 M NaCl) showing the highest slope, this up-turn indicated that all samples contained very large aggregates, in agreement with DLS results.

Due to the limited q range and the polydispersity of protein composition and structure, a quantitative analysis of the SAXS curves was not carried out. Some general structure information of various length scales can still be inferred from the different scattering patterns. The unified fit was conducted to calculate the radius from the scattering curves, thereby predicting the size of related structures (Beaucage, 1995; Ilavsky & Jemian, 2009). As discussed, the sample extracted with 0.0 M NaCl exhibited a higher characteristic length value (around 60 nm) compared to the samples extracted with NaCl which estimated local structures of about 40 nm, indicating that salt extraction caused the presence of a higher amount of smaller local structures.

3.6. Thermal properties

The thermal properties of salt extracted pea protein fractions (0.0 M – 1.2 M NaCl) were measured using NanoDSC. As shown in Fig. 4, three distinct denaturation peaks have been observed in all samples and their denaturation temperature and enthalpy change are shown in Table 4. In the case of water extract, three thermal transition peaks were identified, at 53.9 °C, 76.5 °C, and 86.9 °C. Similarly, three peaks were also noted in the other fractions, but with a shift to higher temperatures in the presence of higher NaCl concentrations. The first thermal transition peak is likely attributed to albumin denaturation. A shoulder observed in the second peak of the water extract may also be related to albumin. Additionally, given the high carbohydrate content (47 %) in the 0.0 M extract and the gelatinization temperature range of pea starch (50–70 °C) (Ratnayake, Hoover, & Warkentin, 2002; Xu & Kuang, 2024), pea starch gelatinization could cause or contribute to both the first peak and the shoulder peak. The second and largest peak, as well as the third peak correspond to the thermal transitions of pea convicilin and vicilin, and legumin, respectively (Hansen, Bu, & Ismail, 2022; Shrestha, van't Hag, Haritos, & Dhital, 2023). Legumin has a higher denaturation temperature than vicilin, due to the compact structure and larger structure, requiring higher level of energy to unfold it. With increasing salt, the two largest peaks were dominating the thermal transition. Furthermore, there was an obvious shift to higher temperature, which increased from 76.5 °C and 86.9 °C at 0.0 M NaCl to 91.1 °C and 106.7 °C at 1.2 M NaCl, respectively. This salt stabilizing effect is consistent with the findings of Sun and Arntfield (2011) and Mession, Sok, Assifaoui, and Saurel (2013). The denature temperature is related to the structure complexity, the more compact structure, the higher level of energy is required to unfold it, resulting in the higher denaturation temperature. Therefore, increased denaturation temperature with salt addition indicated a more compact protein structure, which algins with the SAXS data.

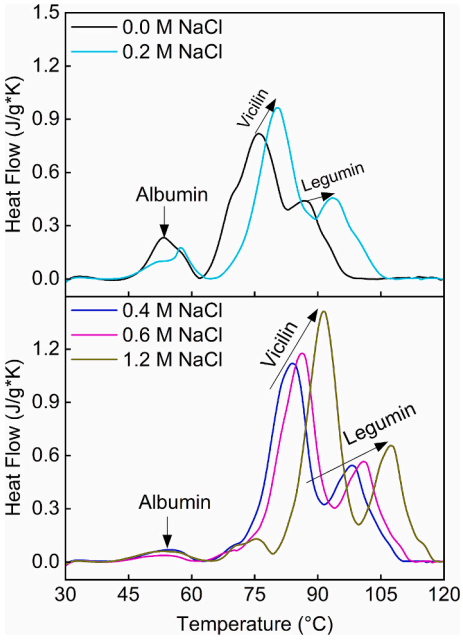

Fig. 4. Heat flow (J/g*K) as a function of temperature measured in the various pea protein solutions extracted with different concentrations of NaCl (0.0–0.2 M, top; 0.4–1.2 M, bottom).

Table 4
Denaturation temperatures T_d and enthalpy change ΔH of different denaturation peaks of pea protein fractions extracted with 0.0–1.2 M NaCl. Values are shown as mean ± standard error (n = 2).

Samples	Peak 1		Peak 2		Peak 3	
	T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)	T_d (°C)	ΔH (J/g)
0.0 M NaCl	53.9 ± 0.0	2.00 ± 0.03	76.5 ± 0.1	8.7 ± 0.5	86.9 ± 0.0[e]	4.4 ± 0.2
0.2 M NaCl	57.4 ± 0.6	1.49 ± 0.01	80.0 ± 0.1	10.3 ± 0.5	93.6 ± 0.0[d]	5.3 ± 0.1
0.4 M NaCl	53.2 ± 0.2	0.92 ± 0.11	83.4 ± 0.2	12.0 ± 0.5	97.6 ± 0.1[c]	5.9 ± 0.1
0.6 M NaCl	53.4 ± 0.8	0.48 ± 0.01	85.8 ± 0.0	13.0 ± 0.1	100.3 ± 0.1[b]	6.0 ± 0.1
1.2 M NaCl	53.8 ± 0.9	0.76 ± 0.04	91.1 ± 0.0	13.6 ± 0.7	106.7 ± 0.1[a]	6.0 ± 0.0

Heat enthalpy changes, determined by the peak area normalized to J/g protein, represent the energy required to unfold proteins during heating, reflecting the extent of structural transitions. The presence of salt decreased the denaturation enthalpy of the first peak, because of the decreased relative ratio of albumin in the extracts. However, this was not the case for pea globulins. Although increasing NaCl concentrations resulted in a higher ratio of legumin to vicilin, it increased denaturation enthalpy of both proteins, suggesting that salt promoted a more ordered protein structure in pea globulins. When comparing samples extracted with 0.4 M and 1.2 M NaCl, which had similar protein compositions (Fig. 1B), vicilin exhibited a steeper heat flow increase than legumin. This suggests that vicilin's structure is more sensitive to salt concentration than legumin, consistent with the SEC-MALS results.

4. Conclusions

Increasing NaCl concentrations during extraction caused important differences in the composition of pea proteins, not only in terms of ratios between albumins, vicilins, and legumins, but also for the presence of carbohydrates and phytates. Extraction of pea proteins with salt concentrations below 0.4 M NaCl, led to different ratios of legumin, convicilin, vicilin, and albumin. At 0.4 M NaCl, protein extractability reached maximum, and the proteins contained high ratio of legumin to vicilin or albumin. Increasing salt concentrations also resulted in less carbohydrates and phytates to protein ratios.

Furthermore, increasing NaCl concentrations during extraction also caused different structure changes in different protein fractions. SEC-MALS results revealed that salt extraction caused the dissociation of pea vicilin and convicilin, as indicated by their lower molar masses at high salt concentrations (0.4–1.2 M NaCl). In contrast, the molar mass of legumin remained unchanged across different salt concentrations. Salt extraction also enhanced the structure compactness of pea globulins, as evidenced by SAXS and NanoDSC results. NanoDSC further revealed that salt exerts a stabilizing effect on globulins, but not on albumins. In addition, increasing salt concentration promoted a higher proportion of ordered structure in pea globulins, with a more pronounced effect on vicilin than on legumin.

In summary, extraction conditions exerted a substantial influence on the extraction efficiency, but also in the composition and structure of the protein extracts. This work clearly demonstrates the importance of ionic interactions between proteins in the concentrates, and it will help design more tailored extraction methodologies to obtain specific compositions and techno-functionality. Although adjusting salt extraction concentrations can selectively extract target proteins with optimal techno-functionality, the industrial relevance of this work is limited, as high sodium should be limited in foods. Therefore, future work should also consider the changes that may occur after salt removal.

CRediT authorship contribution statement

Yi Zhang: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Laura Roman:** Writing – review & editing, Data curation. **Jacob Judas Kain Kirkensgaard:** Writing – review & editing, Data curation. **Milena Corredig:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Yi Zhang reports financial support was provided by China Scholarship Council. Milena Corredig reports financial support was provided by Villum Foundation. Laura Roman reports financial support was provided by Novo Nordisk Foundation. Yi Zhang reports equipment, drugs, or supplies was provided by FOODHAY. If there are other authors, they 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144650.

Data availability

Data will be made available on request.

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