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Impact of Phytase Treatment and Calcium Addition on Gelation of a Protein-Enriched Rapeseed Fraction

Pia Silventoinen[1] · Anni Kortekangas[1] · Emilia Nordlund[1] · Nesli Sozer[1]

Abstract

Rapeseed press cake was upcycled as a protein-enriched ingredient through dry fractionation. The protein-enriched fraction contained higher amounts of phytic acid compared to press cake, and phytase treatment was applied to decrease the phytic acid content from 6.8 to 0.5%. The effect of phytase treatment leading to the release of cations was also mimicked by extrinsic calcium addition. Both phytase treatment and calcium addition significantly improved the heat-induced gel properties but had a minor effect on protein solubility and dispersion stability at pH 8. Water and protein holding capacities of the gels were the highest for the phytase-treated sample (91 and 97%, respectively), followed by the sample with added calcium (86 and 94%, respectively) and control sample (60 and 86%, respectively). Gel firmness followed the same pattern. Scanning electron microscopy images revealed an interconnected structured network in the phytase-treated gel, while in the control gel, a more rigid and open structure was observed. The improved gelation properties resulting from the phytase treatment suggest that the protein and soluble dietary fibre-enriched rapeseed press cake ingredient serve as a promising raw material for gelled food systems. The positive effect of calcium addition on gel properties proposes that part of the improvement observed after phytase treatment may be caused by cations released from phytate.

Keywords Rapeseed press cake · Dry fractionation · Rapeseed protein · Gelation · Phytic acid · Calcium

Introduction

Rapeseed press cake is a potential raw material for sustainable food ingredients with considerable amounts of nutritionally valuable protein and dietary fibre, which is currently, mainly used for feed. In cold-pressed cake, the proteins are in their native state, rendering it an ideal ingredient for food processing. However, usually, the press cakes also include the seed hulls. Cold-pressed cake contains 11–18% residual oil, 32–36% protein, 28% hemicellulose, cellulose, and lignin, 11% sugars and 6% ash, while starch content of mature seeds is negligible (Schöne et al., 1996). As reviewed by Wanasundara (2011), majority of rapeseed proteins (60%) are salt-soluble cruciferins (300 kDa) with pI at 4–7, and the second most abundant protein class (20%) is the water-soluble napins (14.5 kDa) with pI at 11. Rapeseed is rich in phenolic and other bioactive compounds that are often also considered antinutritive factors. One of the antinutrients in the rapeseed press cake is phytic acid (2–5% of defatted cake; Thompson, 1990), which is in intact rapeseed stored throughout the kernel, inside protein storage vacuoles in small globoids, as shown by Yiu et al. (1983) and reviewed by Thompson (1990). It is a negatively charged inositol-phosphate that interacts with positively charged molecules such as cations as well as proteins above their isoelectric points (Cheryan & Rackis, 1980). In rapeseed, proteins exhibit various isoelectric points; thus, protein-phytic acid complexation occurs at all pH values and is suggested to cause technological (Rosa-Sibakov et al., 2018) and nutritional (Cheryan & Rackis, 1980) challenges.

In addition to complexation with other components in the plant matrix, plant proteins often exhibit limited technological functionality compared to animal proteins due to, for example, their low water solubility and presence of insoluble compounds in the protein ingredients. Thus, strategies to improve plant protein performance are considered essential and can include physical and (bio)chemical separation and functionalization technologies

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(Nikinmaa et al., 2018). For example, dry fractionation increases the nutritional value of rapeseed press cake via protein enrichment and diminution of insoluble dietary fibre content (Laguna et al., 2018; Partanen et al., 2016). In general, dry fractionation leads to moderate enrichment of target components such as protein or dietary fibre, thus enabling production of so-called hybrid ingredients which show functionalities related to all components present in the ingredients (Nikinmaa et al., 2018; Silventoinen et al., 2019, 2021). Functionalization by bioprocessing including, for instance, enzymatic treatments has also been widely applied to improve the food applicability of plant-based raw materials (Nasrabadi et al., 2021).

Gelation behaviour is an important characteristic to describe the quality of a plant-based ingredient, since gels are common structures, formed by proteins or polysaccharides commonly via heating or acidification inducing the formation of a continuous network that entraps water, found in foods such as yoghurts and spoonable products. Minimum gelation concentration varies based on raw material, whereas the effect of pH on heat gelation seems to be of the same type in various ingredients: alkaline pH enhances gelation and improves gel properties (Jiang et al., 2014; Kim et al., 2016a, b; Nieto-Nieto et al., 2014). In plant ingredients, not only proteins, but also other components contribute to gel formation, by, for example, shielding protein charge, enhancing phase separation (Message et al., 2013; Nieto-Nieto et al., 2014), or by altering the overall water retention capacity of the matrix (Abdul-Hamid & Luan, 2000; Yang et al., 2018). Especially in the case of charged polysaccharides, such as pectin, ion-induced network formation may improve gelation extensively (Yang et al., 2018).

We have previously shown that phytase treatment of a dry-fractionated protein-enriched rice bran fraction improved heat-induced gelation at alkaline conditions, and the effect was considered to be based on gelation of both protein and dietary fibre (Kortekangas et al., 2020), yet the mechanism of gelation remained to be further studied. The aim of this study was to improve food applicability of a defatted rapeseed cold press cake by dry fractionation and to elucidate mechanisms behind the effect of phytic acid degradation by phytase on the gel-forming ability of the air-classified hybrid ingredient. To unravel these mechanisms, the impact of extrinsic calcium addition prior to gelation was explored. Heat-induced gelation of the hybrid ingredient at alkaline pH was applied and quantitative methods for determination of dispersion and gel properties were employed.

Materials and Methods

Materials

The cold-pressed (50 − 60 °C), pelletized, and air-dried rapeseed press cake raw material was received from Kankaisten Öljykasvit Oy (Turenki, Finland). The initial raw material in the cold oil pressing process was *Brassica rapa* L. seeds. Unless otherwise stated, chemicals were purchased from Sigma (St. Louis, MO, USA).

Dry Processing of Rapeseed Press Cake

The protein-enriched rapeseed press cake fraction was produced by dry fractionation of the rapeseed press cake raw material after fat extraction by supercritical carbon dioxide ($SC-CO_2$). Prior to defatting, the material was pre-crushed in order to facilitate lipid removal. The defatting was carried out using an extraction vessel of 48 L, pressure of 25–30 MPa and temperature of 50–60 °C (NATECO2 GmbH & Co, Wolnzach, Germany). Extraction was carried out in 25 kg batches of rapeseed press cake. During extraction, 12% of press cake was removed. The defatted rapeseed press cake was subsequently milled once using a 100 UPZ mill equipped with pin disc grinders (Hosokawa Alpine AG, Augsburg, Germany) at a rotor speed of 17 800 rpm, and this defatted and pin disc-milled raw material was referred to as the rapeseed press cake (RPC). RPC was air classified with a Minisplit air classifier (British Rema Manufacturing Company Ltd., Chesterfield, UK) at air classifier wheel speed of 5 000 rpm and air flow rate of 220 m³/h. In total nine parallel air classifications were carried out, and average mass yield was reported. In addition, average protein separation efficiency (PSE % dm) was calculated as described in Silventoinen et al. (2021). The obtained fine fractions were combined and the combined fine protein-enriched fraction (PEF) was analysed for its chemical composition and utilized as a raw material in the further steps of this work.

Biochemical Composition and Particle Size

Protein content of the samples was determined based on the total nitrogen content ($N \times 6.25$) using Kjeldahl method as described in Silventoinen et al. (2021). Dietary fibre was quantified enzymatic-gravimetrically according to AOAC method 991.43 (AOAC, 1995). Ash was determined gravimetrically using combustion at 550 °C, and fat content was determined using Soxhlet extraction with heptane for 5 h. The amounts of glucose, fructose, sucrose, maltose, and maltotriose were determined after 30-min water extraction of the samples from the water extracts by high-performance anion-exchange chromatography with pulse amperometric detection (Dionex ICS-3000 equipped with a CarboPac PA1 column; Hausalo, 1995; Sluiter et al., 2008). Phytic acid content was quantified either from raw material as such or freeze-dried dispersions by utilizing a colorimetric reaction between ferric chloride and sulfosalicylic acid based on description of Latta and Eskin (1980) with modifications by Vaintraub and Lapteva (1988). Calcium content of the PEF sample was determined after a

microwave pre-treatment according to DIN EN 13,805 using inductively coupled plasma optical emission spectrometry (EN ISO 11885). All biochemical analyses were carried out at least in duplicates. Particle sizes of RPC and PEF were analysed in triplicates with an Aero S dry module of Mastersizer 3000 (Malvern Analytical, Worcestershire, UK) using Fraunhofer approximation for calculation of the geometric volume distributions.

Dispersion Preparation and Enzyme Treatment

Three PEF-based samples, namely, phytase-treated, 0.005 M Ca^{2+}, and a control dispersion, were prepared. PEF was dispersed in Milli-Q water to achieve a 5% protein concentration and mixed at room temperature for 1 h. NaN_3 at a 0.01% w/v concentration was added to ensure microbial safety throughout processing and storage. The sample containing calcium was prepared by adding an amount of $CaCl_2$, previously optimized for gelation improvement, which corresponded to a 0.005 M Ca^{2+} concentration. The dispersions were then heated to 50 °C and pH was adjusted to 5. A dosage of 100 U g^{-1} dm phytase (source *Aspergillus niger*, optimum pH 4.5–6, UltraBio-Logics Inc., Canada) as a 10% solution (phytase-treated sample) or an equal amount of Milli-Q water (0.005 M Ca^{2+} and the control dispersion samples) was added prior to a 2-h incubation in a 50 °C water bath. After the incubation, the dispersions were cooled to room temperature and pH was adjusted to 8. In the analysis of the dispersions, the enzyme was not inactivated by heat to allow analysis of native dispersion properties. The pH in the following steps (pH 8) was, however, not in the optimal pH range of the enzyme according to the specifications from the manufacturer suggesting clearly reduced activity. These dispersions at pH 8 were used for characterization of dispersion properties and preparation of heat-induced gels. Samples were prepared at least in triplicate.

Dispersion Properties

Dispersion Conductivity

Dispersion conductivity was measured using CDM210 conductivity meter (Radiometer, Copenhagen, Denmark) to assess the impact of ions released during phytase treatment or added as calcium.

Protein Solubility

Determination of protein solubility was performed according to Kortekangas et al. (2020) after centrifugation by quantifying the nitrogen content of the supernatant by Kjeldahl ($N \times 6.25$). Protein solubility (PS) was calculated as the ratio of protein concentration in the supernatant (c_s; mg/mL) to protein concentration in the initial dispersion (c_i; mg/mL), as shown in Eq. (1).

$$\text{Protein solubility}(PS; \%) = \frac{c_s}{c_i} * 100\% \tag{1}$$

Regarding dispersion conductivity and protein solubility, in total, five dispersions were analysed in duplicates.

Dispersion Stability

Analysis of dispersion stability was carried out simultaneously by visual observation and measurement with the optical scanner TurbiScan® LAB Expert (Formulaction SA, France) of the sedimentation at time points 0 h, 1 h, and 24 h. The extracted parameter from the software was the global TSI (Turbiscan Stability Index), which is a dimensionless parameter combining the effect of variations in backscattering and transmission signals of near-infrared light over time. The TSI value represents the dispersion instability: the more the value rises above zero, which represents total stability or stability at the beginning of the measurement, the more destabilization has occurred in the sample. TSI analysis was performed in duplicate for three dispersions.

Gel Properties

For analysis of large deformation properties, water and protein holding capacity of the gels, free thiols, protein solubilization by solvents, and scanning electron microscopy, the heat-induced gelation was conducted in closed tubes or bottles, depending on the analysis, in a water bath at 95 °C for 15 min. Samples were cooled to room temperature and stored in the fridge (+ 6 °C) overnight prior to further analysis.

Small Deformation Oscillatory Measurement

Small deformation oscillatory measurements during heat-induced gelation were performed in a stress-controlled rheometer (AR-2, TA-Instruments, Crawley, UK). The measurement setup comprised a temperature-controlled Peltier plate, a plate-plate geometry (Ø 40 mm), and a solvent trap with rapeseed oil hindering sample from drying. The measurement consisted of a temperature sweep from 25 to 95 °C and back to 25 °C at a constant rate of 2 °C min^{-1} (holding phases of 5 and 15 min at 95 °C and at the end, respectively) in the linear viscoelastic region with constant frequency of 0.1 Hz and strain of 0.5%. Small oscillatory measurements were conducted with five replicates.

Large Deformation

Gel firmness was determined with a Texture Analyser (Stable Micro Systems Ltd., Godalming, UK) equipped with a 5 kg load cell and a cylindrical probe with hemispherical bottom (Ø 1.27 cm). Gels with a 30-mL volume, prepared in 50-mL capped Schott bottles (Ø 46 mm), were analysed directly from the fridge with a one-cycle penetration test with a test speed of 1.00 mm s^{-1} and penetration distance of 15 mm. The extracted value for gel firmness was the maximum force (N) at 10-mm distance after the initial ascent of the force value above 0, i.e., at 10-mm gel depth from the surface.

Water and Protein Holding Capacity

Water holding capacity (WHC) was determined from gels prepared in sealed glass tubes after overnight storage in the fridge. WHC was defined according to Ercili-Cura et al. (2013) and expressed as % of the mass of the pellet remaining after low-speed centrifugation ($3000 \times g$, 10 min, 6 °C) and supernatant removal to the initial gel weight. The released supernatant from WHC was furthermore analysed for protein content to determine the protein holding capacity (PHC) during high-speed centrifugation of the gel. The protein content was determined using Kjeldahl method ($N \times 6.25$) and the PHC was expressed as % of protein remained in the gel (g) after centrifugation when compared to the initial amount of protein (g) in the gel.

Free Thiols

Free thiols of PEF and gels were measured by using the Ellman's reagent according to Nisov et al. (2020). Briefly, reaction buffer consisting of 0.1 M sodium phosphate buffer at pH 8 and 1 mM EDTA, with or without 8 M urea, was mixed with PEF or freeze-dried and ground gel at a ratio of 1 mL to 60 mg. Of this mixture, 250 µl was mixed with 2.55 mL of a previously prepared Ellman's reagent solution (0.008% Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), in reaction buffer). After a 15-min incubation at room temperature, samples were centrifuged ($10\,000 \times g$, 10 min) and absorbance read at 412 nm. A cysteine standard (0.0–1.5 mM) was used to determine free thiols, expressed as cysteine equivalent, in the sample. The amounts of free thiols were determined with and without urea (offering more binding sites to the large Ellman's reagent after breaking hydrogen bonds and partly hydrophobic interactions) and to enable comparison to a non-gelled raw material; also the protein-enriched rapeseed press cake fraction as such, PEF, was analysed.

Protein Solubilization by Solvents

In order to analyse protein interactions of the gel network, gels were solubilized according to Avanza et al. (2004) and Zenker et al. (2020) with modifications. The solutions used as extraction solvents were prepared in a 0.1 M sodium phosphate buffer (pH 8) and contained either 6 M urea (C), 1% (w/v) SDS (D), or a combination of 1% (w/v) SDS, 6 M urea, and 1% (w/v) dithiothreitol (DTT, E); Milli-Q water as such (A) and buffer (B) were also used as extraction solvents. Briefly, 1 g gel was solubilized with 5-mL solvent, mixed for 30 min at room temperature on a swinging mixer and centrifuged ($10\,000 \times g$, 15 °C, 15 min). The protein content of the supernatants was analysed with the RC-DC kit (Bio-Rad Laboratories) in duplicate. Protein solubilization level (%) was calculated as the ratio of protein released into the supernatant after centrifugation (mg) to initial protein content of the gel-solvent mixture (mg). This level served as an indicator for molecular interactions of the gel network.

Scanning Electron Microscopy

Gel microstructure was analysed by scanning electron microscopy (SEM). Gels were frozen in liquid nitrogen and freeze-dried prior to analyses. These dried gels were gently broken by hand to avoid unintentional smoothing of the cross-sectional surface and sputtered with 4 nm Au/Pd (Leica EM ACE200, Germany). The protein-enriched rapeseed press cake fraction (PEF) as such and wetted PEF (mixed at 5% protein concentration for 30 min at room temperature) were also examined. All samples were imaged with a field emission SEM (FE-SEM, Zeiss Merlin, Germany) at an acceleration voltage of 1–2 kV with a probe current of 60 pA. Imaging with SEM was carried out once for each sample.

Statistical Analysis

Statistical analyses were conducted by using IBM SPSS Statistics, Ver. 26 (IBM Corporation, New York, USA). All sample sets were tested for normal distribution of the data based on Kolmogorov–Smirnov and Shapiro–Wilk tests. After this, differences between sample means were assessed by one-way analysis of variance (ANOVA) for normally distributed data and samples were grouped based on post hoc testing with Dunnett's T3 when unequal variances were detected (gel solubilization) and with Tukey's HSD when equal variances were detected (phytic acid content, conductivity, gel firmness, WHC and PHC). For sample data not complying with normal distribution, the Kruskal–Wallis test was employed to detect differences across samples (protein solubility, dispersion stability). Significance level of 0.05 was used in all analyses.

Results and discussion

Dry Fractionation of Rapeseed Press Cake

Dry fractionation consisting of impact milling and air classification increased protein content of the defatted rapeseed press cake (RPC) from 36.0 to 41.9% in the fine protein-enriched fraction (PEF) with a mass yield of 49.0%, which accounted for a 57.0% protein separation efficiency (PSE; Table 1). In addition, the PEF was, as expected, composed of considerably smaller particles than the pin disc-milled raw material, RPC, as indicated by the differences in the particle size distributions (Fig. 1) and median particle sizes of PEF (23.3 µm) and RPC (51.4 µm). In general, proteins are enriched in the finest particles in plant materials, and thus, the protein-rich fractions also typically exhibit small particle size. Contrary to protein, dietary fibre concentrated in the coarse fraction in air classification. The separation of coarse dietary fibres from the proteins led also to lighter colour in PEF (Fig. 1). Previous studies also showed that separation of hulls from the protein fraction in rapeseed press cake reduced the amount of insoluble dietary fibre (IDF) especially cellulose and lignin (Eriksson et al., 1994; Partanen et al., 2016), which are considered to be more resistant to particle size reduction compared to other cellular compartments. On the contrary, soluble dietary fibre (SDF) was slightly enriched in PEF causing also an increase in SDF:IDF ratio from 0.1 to 0.2. Similar results were found also in dry separation of various cereal brans (Silventoinen et al., 2019, 2021). The relative SDF enrichment in PEF resulted from the removal of large amounts of the IDF-rich hulls and enrichment of cell walls rich in soluble dietary fibres, such as pectin (Rommi et al., 2014). In addition to protein and soluble dietary fibre, phytic acid was
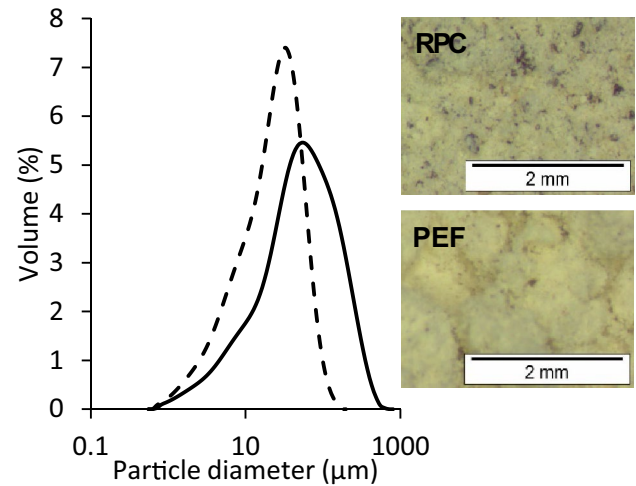
Fig. 1 Particle size distribution and stereomicroscopy images of SC-CO_2-extracted and pin disc-milled rapeseed press cake (RPC; solid line) and protein-enriched fraction produced from RPC by air classification (PEF; dotted line)

also enriched in PEF (6.8 vs 5.8% in RPC). Since phytic acid is known to be located throughout the rapeseed kernel inside the protein bodies (Yiu et al., 1983), its co-enrichment with protein was expected in dry fractionation that relies on size-based separation of particles. Similar phytic acid enrichment to the protein-enriched fraction has been reported in air classification of pulses (Elkowicz & Sosulski, 1982), whereas in cereal brans, even more pronounced enrichment has been noted (Silventoinen et al., 2019, 2021). In the present study, air classification resulted also in enrichment of fat (from 2.2 to 2.4%), ash (from 8.3 to 9.1%), and free sugars (from 12.3 to 14.7%).

Table 1 Biochemical composition of the SC-CO_2-extracted and pin disc-milled rapeseed press cake (RPC) and protein-enriched fraction produced by air classification (PEF). In addition, protein separation efficiency (PSE) and mass yield of PEF are shown

	Defatted and milled rapeseed press cake	Protein-enriched rapeseed press cake fraction
Mass yield (% dm)[a]	-	49.0 ± 1.7
PSE (% dm)[a]	-	57.0 ± 2.6
Protein (% dm)[b]	36.0 ± 0.1	41.9 ± 0.6
Total dietary fibre (% dm)[c]	35.5	26.1
Insoluble dietary fibre (% dm)[c]	32.1	22.4
Soluble dietary fibre[c]	3.4	3.6
Fat (% dm)[b]	2.2 ± 0.1	2.4 ± 0.1
Ash (% dm)[b]	8.3 ± 0.0	9.1 ± 0.0
Free sugars (% dm)[b,d]	12.3 ± 0.3	14.7 ± 0.2
Phytic acid (% dm)[b]	5.8 ± 0.5	6.8 ± 0.03

dm on dry matter basis

[a] ± stdev

[b] ± avedev

[c] Only analysed once

[d] Includes water-soluble monosaccharides and sucrose

Dispersion Properties After Phytase Treatment and Calcium Addition

Phytase treatment decreased the phytic acid content of PEF from 6.0 (control sample) to 0.5% (phytase-treated sample; Table 2). Previously, similar phytase treatment was shown to reduce phytic acid content of rice bran from 22 to 3% (Kortekangas et al., 2020) and a five times lower enzyme dosage reduced the content in faba beans from 1 to 0.1% (Rosa-Sibakov et al., 2018). Phytase treatment leads to degradation of phytate resulting in free divalent cations, phosphates, and inositol phosphates with degree of phosphorylation less than six (Gupta et al., 2013). Impact of extrinsic calcium addition on the PEF properties was studied in this work to reveal the role of released cations during phytic acid reduction. There was no considerable change in the phytic acid content of the incubated control and the 0.005 M Ca^{2+} sample when compared to the content in the original PEF sample.

The phytase-treated sample had the highest electrical conductivity among the three samples (Table 2). This suggests that phytase treatment released more ions compared with the amount of added calcium in the 0.005 M Ca^{2+} sample. Indeed, the analysed calcium content of the PEF was 0.66% dm resulting in more than four times higher calcium concentration of 0.021 M if all of the calcium is considered to be first bound to phytic acid and then released as a result of phytase treatment. Moreover, the amount of added calcium was not high enough to differentiate the sample from the control sample in regard to electrical conductivity. Even though the conductivity values were similar across samples, the ionic strengths of the dispersions probably varied to a higher extent since the 0.005 M Ca^{2+} sample and phytase-treated sample contained more free mono- and divalent cations compared with the control. In addition, the phytase-treated sample also contained other multivalent ions, such as phosphates. These multivalent ions affect conductivity in the same way as monovalent ions, but their impact on ionic strength is in the multiple range (Li & Xiong, 2021).

Protein solubility values of all dispersions (control, phytase-treated, 0.005 M Ca^{2+}) varied between 74 and 76% with no statistical difference. The protein solubility exhibited high values at pH 8 which was expected since that pH is out of the range of the pI's of rapeseed cruciferin (4–7) and napin (11) proteins (Wanasundara, 2011). The high solubility values also support the fact that the gentle supercritical carbon dioxide extraction used for fat removal from the rapeseed press cake did not negatively affect the technological functionality when compared to, for example, commercial processing that includes hexane extraction and toasting and has been shown to result in nitrogen solubility index values as low as 12% (Sun et al., 2008). In this work, protein solubility was presumably also affected by the incubation at pH 5 prior to solubility determination, which caused even higher protein solubility than in previous studies where protein solubility of rapeseed press cake varied between 30 and 70% (Wanasundara et al., 2012). Previously, phytase treatment has increased protein solubility of rice bran at acidic conditions (Kies et al., 2006; Kortekangas et al., 2020). On the contrary, protein solubility and foaming and emulsifying properties of soy proteins have decreased by phytase treatment (Wang et al., 2014), and then again phytate addition has increased protein solubility and improved colloidal stability of a soymilk system (Wang et al., 2018), which may be, at pH above the isoelectric point, associated with the so-called salting-in phenomenon of proteins. However, it must be noted that there are considerable differences in the studied systems depending on whether addition of phytate (e.g. sodium phytate) or removal of natural plant phytates (e.g. enzymatically), has been investigated. In this study, the absence of differences between protein solubility of the control and phytase-treated samples presumably results from the fact that no direct electrostatic interactions between proteins and phytic acid, which could decrease protein solubility, occur at pH values above pI (Bye et al., 2013); thus, protein solubility remained unaffected by phytic acid degradation. It is worth noting, however, that the minor protein class of rapeseed, napin, exhibits an pI in the alkaline range and has been shown to bind to phytic acid through electrostatic interaction at pH values 6–8 (Mothes et al., 1990) forming soluble dimeric complexes. The soluble nature of these napin-phytic acid complexes is the reason why the protein solubility of the control and 0.005 M Ca^{2+} samples (both containing phytic acid) was indifferent to that of the phytase-treated dispersion. Furthermore, in this work, it was evident that the applied addition level of calcium (0.005 M)

Table 2 Phytic acid content, electrical conductivity, protein solubility and dispersion instability (TSI) of control, phytase-treated, and 0.005 M Ca^{2+} dispersions at pH 8. Different letters within the same column indicate significant difference ($p < 0.05$)

|	Phytic acid (% dm)	Conductivity (µS/cm)	Protein solubility (%)	TSI 1 h	TSI 1 d
xControl	6.0 ± 0.2^b	6.9 ± 0.2^a	74.3 ± 1.4^{ab}	0.2 ± 0.1^a	5.8 ± 0.3^a
Phytase-treated	0.5 ± 0.1^a	8.3 ± 0.5^b	75.8 ± 2.0^b	0.3 ± 0.1^a	6.4 ± 0.7^{ab}
0.005 M Ca^{2+}	6.1 ± 0.2^b	7.1 ± 0.1^a	73.7 ± 1.9^a	0.3 ± 0.0^a	7.2 ± 0.8^b

was too low to impair protein solubility (i.e. no salting-out phenomenon occurred).

Understanding the impact of phytase treatment and calcium addition on dispersion stability is crucial since during heat-induced gelation, the raw material dispersion needs to be able to remain stable against fast sedimentation to enable a uniform structure formation during the heating step. All PEF dispersions remained stable during a short observation time as indicated by TSI values of 0.2–0.3 at 1 h (Table 2). The TSI value considers the overall stability of a sample giving insight into microscale changes in sample stability not necessarily visible to the eye. Values below 0.5 express visually excellent dispersions with no significant destabilization (Formulaction, 2021). After 1 day, all dispersions showed destabilization as indicated by TSI values in the range of 3–10 that theoretically correspond to phenomena such as large sedimentation, wide particle size variation, or small phase separation; this destabilization was visually apparent as a minor clarification of the upper part of the dispersion (Fig. 2) and was mostly detected as clarification, whereas sedimentation had a minor role, as measured by the Turbiscan (data not shown). Clarification of the upper part of the dispersions is associated with destabilization mechanisms related to particle size and density (Durand et al., 2003), which is expected for a hybrid material with low oil content and high content of dietary fibre. The TSI value after 1 day was the highest for the sample containing 0.005 M Ca^{2+} (7.2), while the control sample exhibited the lowest value (5.8). Calcium addition has been proposed to deteriorate dispersion stability, most likely through reduction of protein solubility and further contribution to protein aggregation (Manassero et al., 2018). According to J. H. J. Kim et al., (2016a, 2016b), such dispersion instability after ion addition is related to protein charge screening/shielding caused by negative and positive ions. In the current study this phenomenon may have happened due to Cl^- and Ca^{2+} ions added to the solution of 0.005 M Ca^{2+} sample. In the case of phytase-treated samples, however, mainly the release of cations from the negatively charged phytic acid is expected.

Gel Properties Affected by Phytase Treatment and Calcium Addition

Gel firmness exhibited low values for control gels (0.045 N), whereas phytase treatment and calcium addition increased firmness to 0.28 and 0.20 N, respectively (Table 3). Moreover, both phytase treatment and calcium addition improved the ability of gels to hold water and retain proteins under low-speed centrifugation (3000×g; WHC and PHC, respectively). The impact of phytase treatment was more pronounced and led to WHC of 91.3% and PHC of 96.8%, compared with values of 86.3% and 60.1% (WHC) and 94.4% and 85.7% (PHC) for 0.005 M Ca^{2+} and control dispersions, respectively.
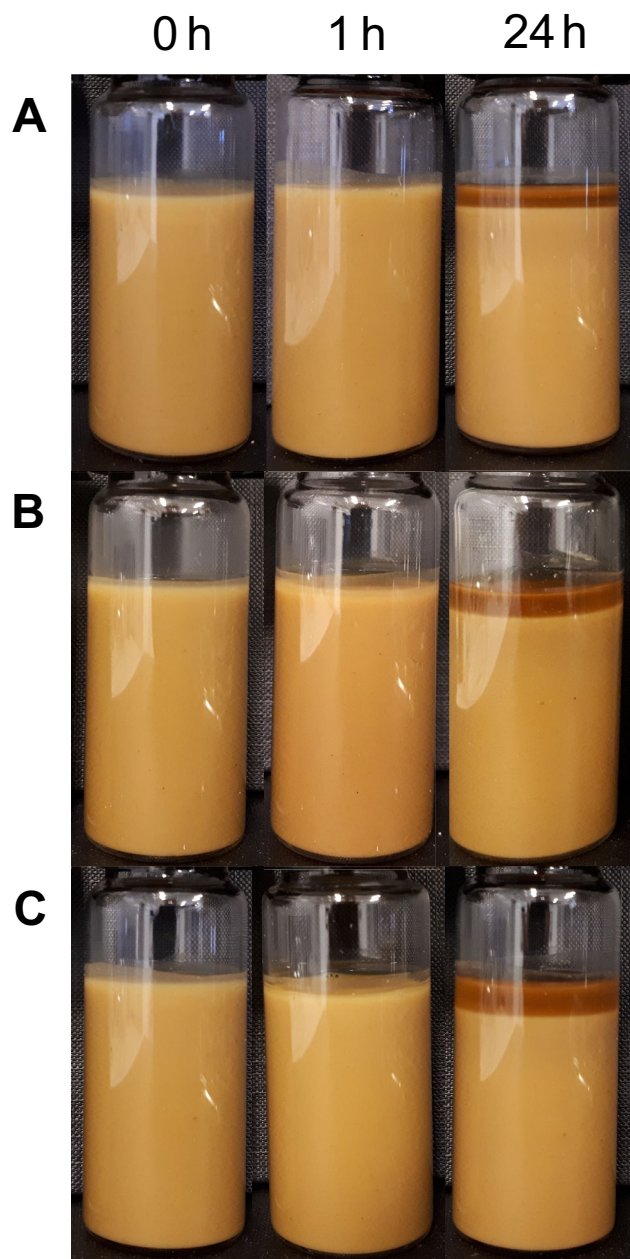
Fig. 2 Images showing dispersion stability of **A** control, **B** phytase-treated, and **C** 0.005 M Ca^{2+} dispersions at time points 0 h, 1 h, and 24 h

We have previously shown that phytase treatment of a rice bran fraction improves WHC of a heat-induced gel at pH 8 (Kortekangas et al., 2020). In the current study, not only the significantly higher gel firmness values of protein-enriched rapeseed gels, but greater water and protein retention ability obtained by both phytase treatment and calcium addition, underline the positive impact of both treatments on gel formation. This result is contrary to the findings of Arntfield (1996) who showed that low levels of phytic acid with or without added calcium (0.01 M $CaCl_2$) improved

Table 3 Gel firmness, water holding capacity (WHC), protein holding capacity (PHC), and amount of free thiols of heat-induced gels prepared from the protein-enriched rapeseed press cake fraction at pH 8 after phytase treatment or calcium addition. Different letters within the same column indicate significant difference ($p < 0.05$)

|	Gel firmness (N)	WHC (%)	PHC (%)	Thiols ($\mu mol/g$)	
				In buffer	In buffer with urea
PEF as such	na	na	na	18.9 ± 0.1	29.3 ± 0.6
Control gel	0.045 ± 0.004^a	60.1 ± 2.0^a	85.7 ± 0.7^a	15.0 ± 0.2	18.7 ± 0.1
Phytase-treated gel	0.28 ± 0.02^c	91.3 ± 1.7^c	96.8 ± 0.6^c	14.1 ± 0.02	21.8 ± 0.2
0.005 M Ca^{2+} gel	0.20 ± 0.02^b	86.3 ± 1.6^b	94.4 ± 0.9^b	14.9 ± 0.3	21.0 ± 0.1

NA Not analysed

properties of heat-induced canola protein isolate gels below the isoelectric point of the protein, whereas at pH 9, no impact on gel firmness was reported with simultaneous phytic acid and calcium addition. Improved heat stability of a soymilk system has also been reported as a result of phytate addition (Wang et al., 2018). In these studies, the effect of phytic acid was evaluated by adding phytic acid to material that was initially free from phytic acid and therefore no release of components (e.g. cations, phosphates) during the treatment took place, which makes comparisons to the here-indicated results challenging. However, Mothes et al. (1990) showed that addition of 0.005 M NaCl decreased the amount of phytic acid bound to napin proteins (leading to breakage of the soluble dimeric napin-phytic acid complexes), which may have facilitated gelation if occurred in the current work. Moreover, phytase treatment has shown to increase the breaking stress of heated soymilk gels and glucono-delta-lactone-acidified soymilk and soy protein isolate gels, which was discussed to potentially result from the released calcium ions having a role in protein gelation (Tsumura et al., 2004). Previously, addition of divalent cations has also shown to improve heat-induced gelation of quinoa protein gels at pH 3.5, while negative impact was observed at pH 7 due to charge screening (Kaspchak et al., 2017), which is contrary to the present findings. However, the level of addition of the divalent cation seems to have a crucial role: in the pre-experiments of the current study, intermediate calcium addition level (0.005 M) enhanced gel firmness and WHC compared with higher or lower addition levels (data not shown). This intermediate calcium addition level was interestingly only one-fourth of the maximum theoretical calcium amount released after phytase treatment (see "Dispersion Properties After Phytase Treatment and Calcium Addition" section). Likewise, Zhao et al. (2018) revealed increase in final G' value after optimal addition level of CaCl₂ (increased when addition increased from 0 to 0.1 M but decreased when concentration reached 0.2 M) and the same was also observed by Lakemond et al., (2003; increased when addition increased from ionic strength I=0.03 to I=0.2 but decreased when ionic strength reached

0.5). In addition, Lakemond et al. (2003) reported decreased amount of non-network protein (i.e. higher protein retention ability) in the soy protein gels after addition of the optimal level of calcium (I=0.2). Despite the increased G' values, both textural properties and WHC of soy protein gels decreased even after the low addition level of CaCl₂ (Lakemond et al., 2003; Zhao et al., 2018), which was contradictory to this study. This suggests that the optimal cation addition levels resulting in improved gel properties vary between different raw materials.

On the contrary to previously mentioned studies on rapeseed protein gelation concentrating on protein isolates, PEF contains, besides proteins, also dietary fibre, which contributes to gelation and final properties of gels. In this study, dietary fibre gelation may have been brought about either through the cations released during phytase treatment or the extrinsically added calcium. We have previously discussed the impact of calcium on pectin gelation (Kortekangas et al., 2020), and this phenomenon, occurring due to calcium-bridge-mediated pectin network formation, may explain the high gel firmness and WHC-values obtained after phytase treatment and calcium addition also in the current study. Furthermore, pectin and/or other network-forming and water-retaining dietary fibres may have improved PHC via entrapment of proteins in the fibre gel matrix inhibiting their release during low-speed centrifugation.

All samples behaved similarly in small deformation oscillatory measurements during heating (Fig. 3) and showed that gel formation, defined as an increase in G' value, was most pronounced during the cooling phase from 95 to 25 °C indicating a notable impact of hydrogen bonds, favoured in conditions with decreased molecular motility, on gel formation (Dickinson, 2012). Final gel strength, i.e. storage modulus G' values, varied for all samples between 1600 and 2000 Pa with no significant differences across samples and high deviations were observed within samples. These deviations may have emerged from the measurement setup comprising a small amount of dispersions with possibly heterogeneous particle sizes. The dynamic nature of oscillatory measurements inhibits its direct correlation with results from
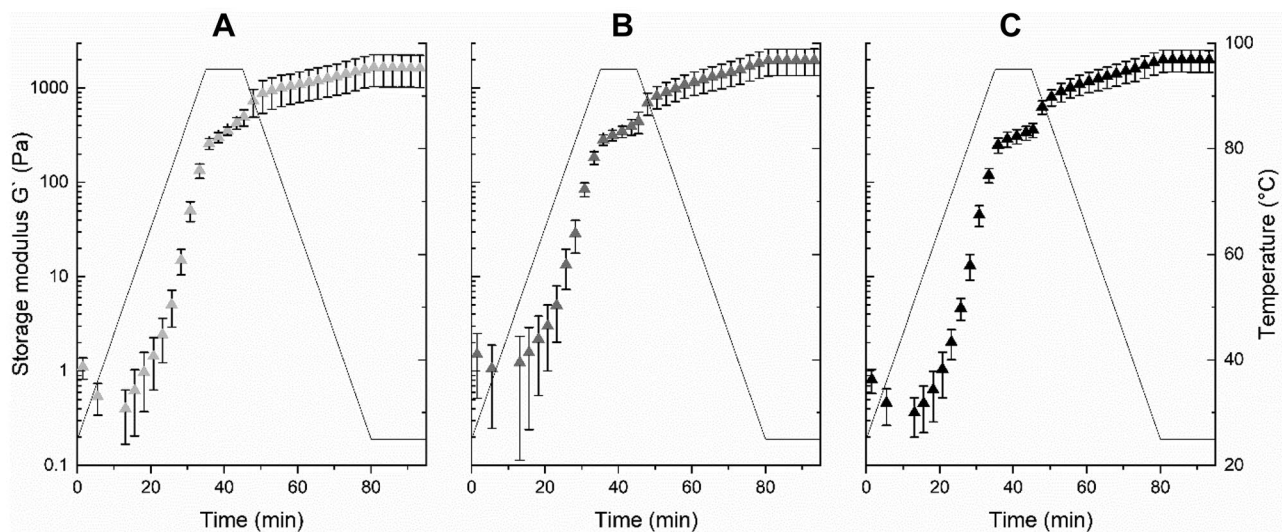
Fig. 3 Development of storage modulus (G') during heat-induced gelation of control **A**, phytase-treated **B**, and 0.005 M Ca^{2+} gels. The solid line represents the applied temperature ramp. Error bars represent standard deviation of five replicates

the static textural measurement of gel firmness where significant differences across the gels were detected (Table 3). Furthermore, the heating step of these samples differed, as rheological measurements were conducted using a relatively slow temperature ramp, whereas all other gel samples were heated at 95 °C only for 10 min. Slow heating may have led to particle sedimentation on a microscale and enabled random molecule migration and aggregation leading to poorly developed gel networks. We have previously shown that phytase treatment of a protein-enriched rice bran improved heat-induced gelation at pH 8 significantly as measured by small deformation oscillatory measurements (Kortekangas et al., 2020). In that material, however, the reduction in phytic acid content was much more pronounced (from 20% in the protein-enriched to 3% in the phytase-treated sample), which may have resulted in stronger impact observed in the small deformation oscillatory measurements of rice bran than rapeseed press cake protein gels.

At alkaline pH, the onset of structure formation in all of the samples of the current study occurred at 65–70 °C (Fig. 3), which is in line with the onset of denaturation measured for canola protein isolate by differential scanning calorimetry (Kim et al., 2016a, b). This onset of structure formation, also referred to as the gelation point, and defined as the cross-over point of the storage modulus G' and loss modulus G'', occurred in all samples at temperatures of approximately 65 °C (data not shown). The employed temperature ramp including temperatures between 25 and 95 °C was chosen based on experimental limitations of going near to the boiling point of water (100 °C) which inhibited the thermal denaturation of napin. At pH 8, the two major rapeseed proteins cruciferin and napin exhibit denaturation temperatures

of 93 °C and 110 °C (Yang et al., 2014), respectively, and higher denaturation enthalpies are reported for napin. It has been shown that cruciferin forms stronger gels with higher gel firmness and springiness compared with napin gels at similar treatment conditions as in the current study (pH 9, 100 °C; Yang et al., 2014). The inclusion of soluble native napin (phytase-treated sample) or soluble napin-phytic acid complexes (control and 0.005 M Ca^{2+} samples; Mothes et al., 1990) in the gels possibly disrupting a continuous network formation may have created further reason for high deviations in rheological measurements.

The level of protein solubilization from the gels in various extraction solvents (Fig. 4) showed no significant differences between the three samples (control, phytase-treated, 0.005 M Ca^{2+}) when each extraction solvent was evaluated independently (data not shown). Likewise, when all three samples were studied as one population to detect the differences between solvents, no differences were observed in the level of solubilization between MilliQ-water (6% of proteins solubilized) and 0.1 M sodium phosphate buffer at pH 8 (7% of proteins solubilized; Fig. 2). All other solvents differed significantly from each other in terms of the protein solubilization level. Addition of 6 M urea or 1% SDS to the buffer increased protein solubilization level to approximately 13% and 25%, respectively, proving the presence of hydrogen bonds and hydrophobic interactions, respectively, in all gel networks. Although these low solubilization levels might indicate a notable effect of disulphide bonds in all gels, the pronounced increase in G' values during cooling but not during heating suggests more the involvement of hydrogen bonds rather than disulphide bonds (Lavoisier et al., 2019). However, the combined effect of urea and SDS with DTT
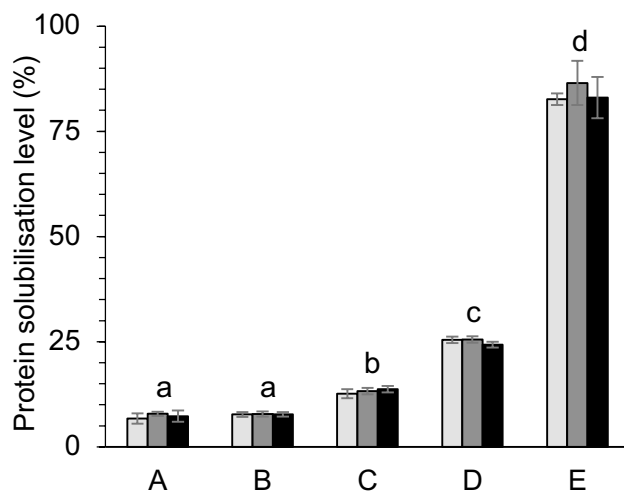
Fig. 4 Protein solubilization level (%) from control (light grey), phytase-treated (dark grey), and 0.005 M Ca²⁺ (black) gels with the aid of solvents: MilliQ-water **A**, 0.1 M sodium phosphate buffer at pH 8 **B**, B + 6 M urea **C**, B + 1% SDS **D**, and B + 6 M urea + 1% SDS + 1% DTT **E**. Statistical difference in protein solubilization level between solvents is indicated as different small letters (p < 0.05)

solubilized up to 87% of gel proteins showing that gel structure was, to a large extent, stabilized by disulphide bonds. The simultaneous addition of chaotropic agents urea and SDS, with DTT, may have enhanced the ability of DTT to reduce disulphide bonds that would otherwise have been buried inside the network stabilized by hydrogen bonds and hydrophobic interactions and similar finding is also reported by Avanza et al. (2004) for amaranth protein gels. This is supported also by Yang et al. (2014) who reported a minimal effect of DTT alone on canola gel properties. Hydrophobic interactions have been shown to be the main molecular interactions in heat-induced gels of rapeseed cruciferin at pH 9, while hydrogen bonds showed highest contribution to napin gels (Yang et al., 2014). However, Perera et al. (2016) showed relatively low surface hydrophobicity values for both rapeseed proteins at pH 7 and 10, which supports our finding of a low impact of hydrophobic interactions in gel formation at alkaline pH. Furthermore, Kim et al., (2016a, b) revealed that no gel was formed by heating a canola protein isolate dispersion containing 5 M urea at pH 7.

The number of free thiols, analysed without breaking the hydrogen bonds and partly hydrophobic interactions with urea, decreased as a result of gelation and the most pronounced decrease was observed for the phytase-treated sample (from 19 to 14 µmol/g). On the contrary, the samples analysed after breaking the hydrogen bonds showed even greater decreasing impact of gelation from 29.3 to

18.7–21.8 µmol/g, and in this case the most pronounced impact of gelation was seen in the control sample. Both of these results suggest the contribution of disulphide bonds, formed by reactions between thiol groups (Monahan et al., 1995), to gelation and thus supports the results on protein solubilization from the gels as discussed earlier. Furthermore, the higher impact of breaking hydrogen bonds and hydrophobic interactions with urea on the raw material compared with gels is also in line with the protein solubilization data that revealed those interactions to have only a minor role in the gels. However, the use of urea most probably improved availability of the free thiols to Ellman's reagent as has been earlier discussed by Nisov et al. (2020). The differences in the numbers of free thiols between the gel samples remained rather low, which complicates drawing conclusions on the importance of disulphide bond formation in differently treated gel samples. Additionally, oxidation of thiol groups during the measurement was not specifically prevented which may have resulted in lower free thiol contents than expected.

Scanning electron microscopy images revealed an interconnected structured network in the phytase- and 0.005 M Ca²⁺–treated gels, while in the control gel a more rigid and open structure was observed (Fig. 5C–E). All gels showed phase-separated areas where dense sheet-like structures formed a continuous network with particulate matter in and around the sheets. Phytase treatment resulted in the formation of a honeycomb-like structure, characteristic for pectin/fibre-based gels (Yang et al., 2018). This structure is absent in gels with added calcium, suggesting a more pronounced effect of the phytase treatment compared with the addition of calcium alone on fibrous parts present in PEF. Due to the fact that a part of the soluble dietary fibre of rapeseed is composed of pectin (Müller-Maatsch et al., 2016), gelation of pectin in the presence of calcium can also be considered. Rapeseed pectin is mostly composed of low-methoxyl (LM) pectin with a degree of esterification below 50% (Müller-Maatsch et al., 2016). LM pectin may, in heat-induced gelation, form a compact, uniform and honeycomb-like structure at alkaline (pH 8.5) conditions (Yang et al., 2018). Comparison of the gel microstructures with the dry and wetted PEF revealed that chemical interactions leading to a set structure arise in heat-induced gelation (Fig. 5A–B vs C–E). The dry protein-enriched rapeseed press cake fraction consisted of particulate matter that, once wetted, swelled, and formed an interconnected matrix with sheet-like microstructure (Fig. 5A–B). At this point, the connected matrix was mostly formed through physical interactions of swollen fibre chains and the appearance changed when the samples were gelled.
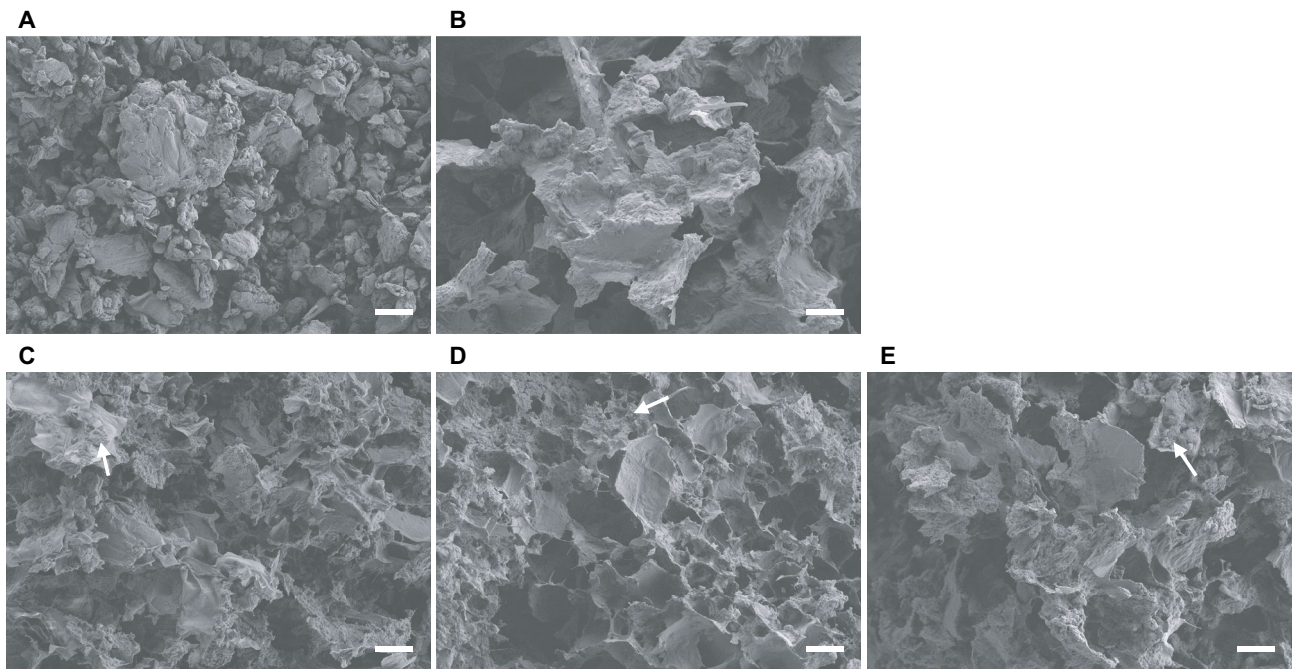
Fig. 5 SEM images of protein-enriched rapeseed press cake fraction, PEF, as such **A**, wetted PEF **B**, control **C**, phytase-treated **D**, and 0.005 M Ca^{2+} **E** gels. Scale bars represent 10 μm. White arrows indicate the particulate matter

Conclusions

Dispersion properties of protein-enriched rapeseed press cake fraction showed no major differences between phytase-treated, 0.005 M Ca^{2+} and control PEF sample. Significant differences in conductivity values of the phytase-treated sample compared with the other dispersions, as well as assumed minor differences in free ions, were not enough to affect protein solubility via salting-in/-out phenomena.

Major differences were noticed in the gel properties of the differently treated PEF samples. Both phytase treatment and addition of extrinsic calcium improved gel firmness and water and protein holding capacities. Overall, the phytase-treated sample showed the highest values. The observed findings are suggested to result from various factors. First, gelation of pectin is known to occur at pH 8 during cooling in the presence of calcium (Yang et al., 2018). The most honeycomb-like structure, distinctive for pectin gels, was observed for the phytase-treated sample in SEM images, which supports the somewhat better gel properties (gel firmness, water and protein holding capacities) compared with the 0.005 M Ca^{2+} gel. Contrary to gel strength and abilities of the gels to retain water and protein, the molecular interactions between different gel samples did not show significant differences. This may further support the fact that gelation of the protein-enriched rapeseed press cake ingredient is largely affected by the fibre components (e.g. pectin) rather than protein.

Second, the positive impact of the increased free calcium content on heat-induced gelation of the studied PEF samples may also result from contribution of calcium to protein gelation. As discussed earlier, various studies on plant proteins have indicated that optimal level of calcium addition may favour protein gelation (Lakemond et al., 2003; Zhao et al., 2018). Indeed, microstructural observations reveal that the protein gelation may have played the major role in gelation of the 0.005 M Ca^{2+} sample.

Third, even though the impact of calcium addition on protein charge screening was not detected in the dispersion properties, it may have played a role in gelation. Differences in the amounts of free ions and possibly higher ionic strength may partly explain the slightly more improved gel properties observed for the phytase-treated sample compared with the 0.005 M Ca^{2+} sample. Furthermore, the higher amount of calcium potentially liberated as a result of phytase treatment (when calculated based on the calcium content of the sample) as well as liberation of other divalent cations from phytate complexes during phytase treatment, may have resulted in improved gel properties of the phytase-treated sample when compared with the 0.005 M Ca^{2+} sample.

In the current work, both phytase treatment and addition of extrinsic calcium improved heat-induced gelation of a protein-enriched rapeseed press cake hybrid-ingredient. The results underline that gelation of both protein and dietary fibre play a role in the gel matrix formation. Functionalization of the protein-enriched rapeseed press cake fraction by

either phytase treatment or adding calcium may find applicability in gelled food systems and facilitate use of the sustainable side-stream-based ingredient containing good amount of nutritionally important protein and dietary fibre.

Abbreviations PEF: Protein-enriched rapeseed press cake fraction; PHC: Protein holding capacity of the gel; PS: Protein solubility; PSE: Protein separation efficiency; RPC: Rapeseed press cake, defatted, pin disc-milled; SC-CO$_2$: Supercritical carbon dioxide extraction; WHC: Water holding capacity of the gel; SDS: Sodium dodecyl sulphate; DTT: Dithiothreitol

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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