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Impact of extraction conditions and seed variety on the characteristics of pennycress (*Thlaspi arvense*) protein: a structure and function approach

Rachel Mitacek[1] | **M. David Marks**[2] | **Nicole Kerr**[1] | **Daniel Gallaher**[1] | **Baraem P. Ismail**[1]

[1]Food Science and Nutrition Department, University of Minnesota, Saint Paul, Minnesota, USA

[2]Department of Plant and Microbial Biology, University of Minnesota, Saint Paul, Minnesota, USA

Correspondence
Baraem P. Ismail, Food Science and Nutrition Department, University of Minnesota, 1334 Eckles Avenue, St. Paul, MN 55108, USA.
Email: bismailm@umn.edu

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Abstract

As the consumer demand for plant proteins continues to grow, the food industry is seeking novel and sustainable protein sources to incorporate in various food products. Pennycress (*Thlaspi arvense*), a sustainable cover crop, produces oilseeds high in protein, warranting investigation. Accordingly, protein extraction from pennycress was evaluated under various extraction conditions, using alkaline extraction and salt solubilization coupled with ultrafiltration. Given the superior color and functionality of the salt extracted pennycress protein isolate (PcPI), its production was scaled-up about two hundred folds in a pilot plant. Furthermore, a new pennycress accession bred to have zero erucic acid (0EA) was evaluated to determine the impact of seed variety on protein characteristics. Structural and functional characterization was performed on PcPI and compared to native (nSPI) and commercial (cSPI) soy protein isolates. Salt extracted PcPI had comparable gel strength to cSPI, three times higher solubility under acidic conditions, and ~1.5 times better emulsification capacity. PcPI extracted from 0EA was mildly different in structure and functionality from that extracted from wildtype pennycress, with the slight variation attributed to genetic variance. Finally, the protein digestibility-corrected amino acid score (PDCAAS) of the salt extracted PcPI, calculated in vivo (0.72) and in vitro (0.87), was superior or comparable to other plant protein sources. This research provided, for the first time, a comprehensive evaluation of different protein extraction protocols to produce a functional PcPI that can compete with soy protein for various food applications, such as acidic beverages, meat and dairy products, and emulsified systems.

KEYWORDS
pennycress protein isolate, protein extraction optimization, protein nutritional quality, protein structure and functionality

INTRODUCTION

The world population in 2020 was 7.8 billion and is projected to reach 9.7 billion in 2050 (United Nations Department of Economic and Social Affairs, 2021). With this growth in population comes a need to enhance food production on the land. However, arable land area is rapidly decreasing, necessitating improvements in the productivity of agricultural practices. One way that farmers can increase crop production is to implement multiple cropping systems utilizing cover crops. Cover crops can integrate well with conventional annual crops (e.g., corn/soybean) and summer cropping systems, replenishing and protecting the soil, and decreasing the length of fallow periods (Clark, 2015). However, farmers will be reluctant to plant a crop if there is not a strong

market, and this market pull cannot be established unless the crop is developed for end use, namely food applications.

Pennycress (*Thlaspi arvense*) is a winter cover crop that has numerous environmental benefits including soil stabilization, nutrients sequestration, and reduced nitrate leaching (Clark, 2015). Pennycress is an oilseed crop of the *Brassicacae* family, closely related to canola and camelina (Warwick et al., 2002). Pennycress oilseed is high in fat (30%–40%) and protein content (25%–35%), thus it presents an attractive choice for both oil and protein ingredients production. Oil from pennycress seeds is currently utilized for industrial biofuels (Moser, 2012). After oil extraction, a proteinaceous meal remains. Extracting protein from the meal provides an opportunity to increase the crop value and incentivize farmers to grow this sustainable cover crop. Additionally, developing protein ingredients from pennycress oilseeds addresses the consumer's increasing demand for novel and sustainable sources of plant protein in their diets (Grand View Research, 2021; Ismail et al., 2020). The global plant protein market was valued at $13.18 billion in 2021 and was estimated at $14.58 billion in 2022 (Meticulous Market Research, 2022).

Research on the utilization of pennycress oilseeds for food applications is limited due to the association of antinutrients, namely erucic acid and glucosinolates. Pennycress oilseeds contain a high amount of erucic acid (~31%–35% of the oil), a fatty acid associated with adverse health effects (Badawy et al., 1994; Liu et al., 2022; Warwick et al., 2002). However, agricultural advancements and screening of chemically mutagenized pennycress has led to the identification of accessions with no erucic acid, suitable for human consumption (Chopra et al., 2019). In addition, pennycress oilseeds, similar to other Brassica family crops, are abundant in glucosinolates, which are secondary metabolites associated with toxic effects such as thyroid, kidney, and liver abnormalities (Tripathi & Mishra, 2007; Warwick et al., 2002). However, Vaughn et al. (2006) reported that pennycress oilseeds only contain the glucosinolate sinigrin, which is not toxic but is responsible for astringent flavor as in other Brassica family crops, such as mustard and horseradish. Glucosinolates are enriched in the meal after oil extraction; however, during protein extraction and concentration most of the glucosinolates are lost (Hojilla-Evangelista et al., 2015).

Different protein extraction methods have been investigated to produce pennycress protein isolate (PcPI). The most common extraction method for the production of isolates is alkaline solubilization coupled with isoelectric precipitation, referred to as alkaline extraction (del Mar Contreras et al., 2019). Alternatively, proteins can be extracted and concentrated following a salt extraction approach (Kumar et al., 2021). Commonly, salt extraction involves solubilization of the protein using a dilute salt solution followed by concentration using high amount of salt (salting out; Nehete et al., 2013). Ultrafiltration has also been investigated as a means of concentration instead of salt precipitation, to avoid the need for excessive water use and the generation of high amount of waste (Tan et al., 2011). However, this approach needs to be evaluated for scale up feasibility. While both alkaline and salt extraction methods have been investigated for PcPI production (Hojilla-Evangelista et al., 2014, 2015), extraction conditions (e.g., pH, ionic strength, time, and number of solubilizations) need further optimization for high protein yields and purity.

Extraction yield is significant from an economic standpoint, whereas high protein purity is relevant when considering the ingredient's nutritional contribution as well as functional properties such as gelation, water holding, emulsification, and foaming in different food applications (Ismail et al., 2020). Both functionality and nutritional quality of plant proteins are governed by environmental and processing conditions. Limited research has explored the impact of different protein extraction conditions on the amino acid composition, protein profile, and functional properties of PcPI (Hojilla-Evangelista et al., 2014, 2015). PcPI structural properties in relation to functionality and nutritional quality need further exploration. It is crucial to not only investigate the impact of extraction method on the structural and functional characteristics of PcPI, but to also evaluate scaling up production of the isolate to better relate industrial processing to ingredient behavior in food applications. Additionally, in this unique crop, breeding is a key factor in the success of its incorporation in food products. Along with the breeding efforts to reduce antinutrients, the impact on the protein's characteristics should not be overlooked. Therefore, the objectives of this study were to (1) optimize protein extraction conditions to maximize yield and purity following two extraction methods, alkaline solubilization coupled with isoelectric precipitation and salt solubilization coupled with ultrafiltration; and (2) characterize structural, functional, and nutritional properties of PcPIs as impacted by the extraction method, scaling up, and difference in variety.

EXPERIMENTAL PROCEDURES

Materials

Wild-type (W) pennycress seeds (*Thlaspi arvense* line MN106) were previously described by Dorn et al. (2013) and originally collected in Coates, MN. Zero erucic acid (0EA) pennycress seeds (*Thlaspi arvense* line fae1-1) were previously described by Chopra et al. (2020), and developed using classical mutagenesis with ethyl methane sulfonate (EMS; Chopra et al., 2018). Wild-type and 0EA pennycress seeds were bulk planted in fall 2016 and harvested in summer 2017 from research fields at

the University of Minnesota (St. Paul, MN, USA). Commercial defatted soy flour (Nutrisoy 7B, 53% protein) and soy protein isolate (cSPI, ProFam® 974, 90.7% protein, 2.36% ash) were kindly provided by Archer Daniels Midland (ADM; Decatur, IL, USA). Sudan Red 7B was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Criterion™ TGX™ 4%–20% precast gels, Laemmli sample buffer, 10× Tris/Glycine/sodium dodecyl sulfate (SDS) running buffer, Imperial™ Protein Stain, immobilized pH gradient (IPG) strips (ReadyStrip 11 cm, pH 3–10, linear), Biolyte 3–10 pH ampholyte, 4%–20% acrylamide Criterion TGX gels with 11 cm IPG + 1 well, ReadyPrep equilibration buffer II, and Precision Plus molecular weight marker were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Vivaflow® membrane ultrafiltration (UF) cassettes (3 kD MWCO) were purchased from Sartorius™ (Gottingen, Germany). 8-Anilino-1-napthalenesulfonic acid ammonium salt (ANS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemical grade reagents were purchased from either Thermo Fisher Scientific or Sigma-Aldrich.

Production of native soy protein isolate

Native soy protein isolate (nSPI) was extracted from soy flour (200 g) following alkaline solubilization (10% total solids) followed by isoelectric precipitation as outlined by Margatan et al. (2013), with the modification of dialyzing (using 3.5 kD MWCO dialysis tubing) the redispersed, neutralized protein fraction prior to lyophilization. Protein content (94.3% protein) of the lyophilized nSPI was determined following the Dumas method (AOAC 990.03; conversion factor of 6.25) using a LECO® FP 828 nitrogen analyzer (LECO, St. Joseph, MI, USA). Ash content of nSPI (3.5% ash) was determined following the official AOAC dry ashing method (AOAC 942.05).

Preparation of defatted pennycress meal

Pennycress seeds (W and 0EA) were screw-pressed with a M70 Oil Press (AgOilPress©, Eau Claire, WI, USA) at 55°C through a 10 mm die. The pressed pellets were ground in a food processor with liquid nitrogen for 1 min. The coarsely ground meal (60 g at a time) was then mixed with hexane (1:3 wt/vol) at 350 rpm for 30 min at ambient temperature. The mixture was centrifuged at 5000*g* for 10 min, the supernatant was poured off, and the meal was air-dried under a fume hood overnight. The dried meal was then milled to 60-mesh using an UDY sample mill (UDY Corporation, Fort Collins, CO, USA). Milled meal (60 g at a time) was subjected to two rounds of 30-min hexane (1:3 wt/vol) extractions to remove residual oil and dried overnight under the hood. The fat content of W-DPM and 0EA-DPM was 3.0% and 3.6%, respectively, as determined by the Mojonnier method (AOAC 922.06). The protein content of W-DPM and 0EA-DPM was 29.6% and 36.0%, respectively, as determined by the Dumas method.

Optimization of protein extraction by alkaline solubilization coupled with isoelectric precipitation

Protein was extracted from W-DPM using the method outlined by Boyle et al. (2018) for camelina oilseeds, with modifications. W-DPM was either subjected to an initial degumming step or left without degumming. For the degumming step, W-DPM (5 g) was solubilized in distilled and deionized water (DDW; 2.5% wt/vol) for 1 h at room temperature, then centrifuged at 12,000*g* for 20 min, the supernatant was discarded, and the pellet was retained for alkaline solubilization. W-DPM, or the retained pellet post degumming, was dispersed in DDW (5% wt/vol) for either 1 or 2 h at either pH 10, 11, or 12 using 2 M NaOH, then centrifuged at 12,000*g* for 15 min. The residual pellet was either lyophilized for protein content measurement or redispersed at 5% solids at either pH 10, 11, or 12 using 2 M NaOH for either 1 or 2 h, and once again centrifuged at 12,000*g* for 15 min. Supernatants were collected and soluble proteins were precipitated by adjusting the pH to 5 with 2 M HCl, followed by centrifugation at 12,000*g* for 10 min. The resulting protein pellet was dispersed in DDW (1:4 wt/vol), neutralized to pH 7 using 2 M NaOH, dialyzed using 3.5 kD MWCO dialysis tubing against DDW at 4°C to remove salts, and lyophilized. Protein and ash contents of the lyophilized samples were determined following Dumas and dry ashing, respectively, as described in the preceding sections. Mass balance and protein purity were utilized to calculate protein yield. Protein purity and yield were used to determine the effectiveness of the tested extraction conditions. Optimal protein extraction conditions were selected based on yield and purity and were utilized to test the inclusion of either 0.1%, 0.2%, or 0.4% sodium sulfite during the initial alkaline solubilization to mitigate oxidation at elevated pH. A larger amount of PcPI was produced from W-DPM (60 g at a time) under the optimal conditions without sodium sulfite (W-PcPI-pH; 89.0% protein, 2.4% ash), and with sodium sulfite (W-PcPI-pH-S; 89.6% protein, 1.9% ash) for protein structural and functional characterization. Extractions were performed in triplicate and samples were stored at −20°C.

Optimization of protein extraction by salt solubilization coupled with ultrafiltration

Protein extraction from W-DPM using salt solubilization coupled with membrane filtration was based on the method reported by Hojilla-Evangelista et al. (2015),

with modifications. The utilization of ultrafiltration (UF) for protein purification required an initial degumming step to mitigate membrane fouling, as described in the previous section using 5 g of W-DPM. Water soluble proteins in the supernatant from the degumming step were precipitated at pH 3 followed by centrifugation at 12,000*g* for 15 min, and the precipitate was set aside for later downstream mixing. The retained pellet post degumming was dispersed in either 0.5, 0.75 or 1 M NaCl (5% wt/vol) with no pH adjustment or with pH adjustment to pH 7 or 8 and stirred for 1 h in a 50°C water bath, followed by centrifugation at 12,000*g* for 20 min. The pellet was either lyophilized for protein content measurement or redispersed in either 0.5, 0.75, or 1 M NaCl (5% solids wt/vol) with either no pH adjustment or with pH adjustment to pH 7 or 8 and stirred for 1 h in a 50°C water bath, followed by centrifugation at 12,000*g* for 20 min. Based on preliminary trial, heating at 50°C compared with room temperature enhanced the salt solubilization of the protein. The supernatants from both solubilization cycles were combined, to which the protein precipitate from degumming step was then added. The mixture was then neutralized to pH 7 with 2 M NaOH. The neutralized solution was subjected to crossflow (tangential) UF using a benchtop Sartorius Vivaflow® 200 system with two Vivaflow® 200 cm^2 membrane cassettes with a 3 kD MWCO (Sartorius™, Gottingen, Germany) running in parallel following the procedure described by Hansen et al. (2022). The ultrafiltered protein solution was either lyophilized or dialyzed against DDW at 4°C using 3.5 kD MWCO dialysis tubing to further reduce the salt content and enhance the protein purity, and then lyophilized. Protein and ash contents of the lyophilized samples were determined following Dumas and dry ashing, respectively, as previously described. Mass balance and protein purity were utilized to calculate protein yield and determine the effectiveness of each extraction condition. Larger amounts of W-PcPI-Salt (92.3% protein, 6.3% ash) and 0EA-PcPI-Salt (90.7% protein, 6.1% ash) were produced from W-DPM and 0EA-DPM (60 g at a time), respectively, under the selected optimal conditions (two 1-h solubilizations in 0.5 M NaCl at pH ~6.3, with dialysis), for protein structural and functional characterization. Extractions were carried out in triplicate and samples were stored at −20°C.

Pilot plant scale-up of protein extraction by salt solubilization coupled with UF

Salt extraction conditions that resulted in optimal yield and purity were scaled up (190 folds) in the pilot plant. Modifications were necessary to adapt the extraction process to the pilot plant equipment, as would be expected. Briefly, W-DPM (25 lbs. equivalent to 11.4 kg) was dispersed in deionized (DI) water (2.5%, wt/vol) and agitated in a jacketed tank with an automated stirrer for 1 h. The dispersion was separated using a desludging disc centrifuge (Westfalia SB7, 1 gal/min, GEA Westfalia Separator Group Gmbh, Oelde, Germany). The wet precipitate was weighed, and percent total solids (%TS) was measured using a CEM AVC-80 Microwave Moisture Balance Analyzer (CEM, Charlotte, NC, USA) to determine the volume of 0.5 M NaCl needed to resuspend the precipitate at 5% solids (wt/vol). The precipitate was dispersed in the determined volume of 0.5 M NaCl and agitated in the same jacketed tank (150 gallon) for 1 h at 50°C. The supernatant retained from the disc centrifuge separation was adjusted to pH 3 with 4.5 N HCl to precipitate soluble proteins. The solution was then separated using the disc centrifuge and the protein precipitate was weighed, resuspended in DI water (1:4, wt/vol), neutralized to pH 7 with 6.25 N NaOH, and saved. The remaining dispersion in 0.5 M NaCl was further separated using the disc centrifuge. After weighing and measuring the %TS of the precipitate, it was redispersed (5% wt/vol) in 0.5 M NaCl and agitated in the same jacketed tank for another 1 h. The dispersion was separated using the disc centrifuge, and the pellet was discarded. Supernatants from the two salt solubilization cycles were combined with the resuspended protein from the degumming step, neutralized with 6.25 N NaOH, and stored overnight at 6–8°C. The solution then underwent UF/diafiltration using a UF/reverse osmosis (RO) unit (15–20 psi inlet, 10–15 psi outlet, PTI Advanced Filtration, PTI Technologies, St. Louis, MO, USA) with tangential cross flow and a spiral wound membrane (3 kD MWCO), until a zero %TS in the permeate was reached. The retentate was pasteurized using a high temperature short time (HTST; 73°C for 15 s) MicroThermics processing system, followed by a two-stage homogenization (Gaulin 125 L, 2500 psi, 60 gal/h, Manton-Gaulin Mfg. Co. Inc., Everett, MA, USA), and spray drying using a SPX Flow Anhydro Spray Dryer (180°C inlet, 90°C outlet, 2.4 gal/h) with a wheel type atomizer (24,500 rpm; SPX Flow, Inc., Charlotte, NC, USA). Protein and ash contents of the spray dried sample were determined following Dumas and dry ashing, respectively, as described previously. The scaled-up W-PcPI-Salt (W-PcPI-SU-Salt; 84.4% protein, 6.8% ash) was stored at −20°C until use.

Color analysis

Color measurements of commercially obtained cSPI and all extracted protein isolates were done in triplicate using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan) as described by Bu et al. (2022). To assess the effect of sodium sulfite inclusion during alkaline extraction, the total color difference (ΔE) between the alkali extracted PcPI samples with and without the use of sodium sulfite was calculated.

Protein profiling by gel electrophoresis

Protein profiling and imaging were performed using one-dimensional (1D) SDS-PAGE and 2D gel electrophoresis as outlined by Boyle et al. (2018). For 1D SDS-PAGE, protein samples (5 μL; containing ~50 μg protein) prepared in Laemmli buffer, and Precision Plus™ MW standard (10 μL) were loaded onto a Criterion™ TGX™ 4%–20% precast Tris–HCl gradient gel. For 2D gel electrophoresis, protein samples (0.25 μg protein/mL) were solubilized in isoelectric focusing (IEF) buffer then electrophoresed using pI 3–10 linear IPG strips as described previously (Boyle et al., 2018). The focused strip was placed in an 11 cm well of a 4%–20% TGX criterion IPG + 1 well gel, along with 7 μL of molecular weight standard marker. The gels were electrophoresed, stained, destained, and imaged as previously described by Boyle et al. (2018).

Molecular weight distribution by size-exclusion high performance liquid chromatography

The molecular weight distribution of the protein isolates was evaluated by size-exclusion high performance liquid chromatography (SE-HPLC) as outlined by Brückner-Gühmann et al. (2018) and modified by Bu et al. (2022), using a Shimadzu HPLC system (Shimadzu Scientific Instruments, Colombia, MD, USA) equipped with and a Superdex 200 Increase 10/300 GL Prepacked Tricorn™ Column, SIL-10AF auto injector, LC-20AT pump system, CTO-20A column oven, SPD-M20A photodiode array detector, and a CBM-20A communication Module. Samples (1% protein, wt/vol), in triplicate, were solubilized for 2 h at room temperature in three different sample buffers: (1) phosphate buffer (0.05 M sodium phosphate buffer, pH 7; 0.1 M NaCl); (2) SDS phosphate buffer (0.05 M sodium phosphate buffer; 0.1 M NaCl; 0.1% SDS); and (3) SDS/β-mercaptoethanol (BME) buffer (0.05 M sodium phosphate buffer; 0.1 M NaCl; 0.1% SDS; 2.5% β-mercaptoethanol), to evaluate the molecular weight distribution, degree of polymerization, and association of proteins through noncovalent and covalent interactions. To avoid clogging the column, insoluble aggregates were filtered out by passing the samples through a 0.45 μm filter. Analyses and detection were performed as described by Bu et al. (2022).

Thermal denaturation by differential scanning calorimetry

Protein denaturation temperature and enthalpy were analyzed, in triplicate, using a DSC1 instrument (Mettler-Toledo, Columbus, OH, USA) and carried out as described by Boyle et al. (2018). Thermograms were manually integrated to determine denaturation temperature and enthalpy using Mettler Toledo's STARe software version 11.00.

Measurement of protein surface properties

Surface hydrophobicity was determined fluorometrically using ANS probe following the method described by Boyle et al. (2018) and modified by Bu et al. (2022). Protein surface charge was determined by measuring zeta potential using a dynamic light scattering instrument (Malvern Zetasizer-Nano ZS, Malvern Panalytical, Cambridge, UK). Zeta potential was calculated by Malvern's Zetasizer software (version 7.13) using the Smoluchowski model. Surface properties were determined in triplicate.

Secondary structure analysis by attenuated total reflectance Fourier transform infrared spectroscopy

Protein secondary structures were assessed, in triplicate, using attenuated total reflectance Fourier transform infrared spectra (ATR-FTIR) as described by Bu et al. (2022). ATR spectra were collected and converted to transmission spectra using OMNIC® software. Second derivative of Amide I band (1600–1700 cm^{-1}) were obtained by PeakFit v. 4.12 to identify α-helix, β-sheet, β-turn, and random coil distribution.

Protein solubility

Protein solubility was measured following the method outlined by Boyle et al. (2018). Protein solutions (1% protein in DDW, wt/vol) were prepared in triplicate and solubilized for 2 h on a magnetic stirrer. Protein solubility was measured at pH 3.4 and 7 under non-heated and heated conditions to simulate acidic and neutral beverage systems, with nSPI and cSPI serving as references. Samples were adjusted to pH 7 or pH 3.4 using 2 N NaOH or 2 N HCl and were assessed at room temperature and after heat treatment (80°C for 30 min). Protein solubility was expressed as the percentage of soluble protein in the supernatant after centrifugation at 15,682*g* compared with the total protein content in the initial solution as determined by the Dumas method.

Water holding capacity and gel strength

The water holding capacity (WHC) and gel strength of thermally induced gels were determined as described by Boyle et al. (2018). Protein solutions (15% protein in DDW, wt/vol) were prepared in triplicate at pH 7 and

stirred on a magnetic stirrer at ambient temperature for 2 h. Samples were heated in a water bath set at 95°C for 10 min, followed by cooling to room temperature (for 2 h) to form gels. WHC was expressed as the percent of water physically entrapped in the gel matrix. Gel strength was measured using a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) equipped with a 100 mm diameter probe at 0.17 mm/s test speed and a target distance of 0.5 mm from the plate. The maximum force as measured in Newtons indicated rupture force of the gel.

Emulsion capacity

Emulsion capacity (EC) was determined, in triplicate, at 1% protein (wt/vol in DDW) as described by Boyle et al. (2018) and modified by Bu et al. (2022). Emulsification capacity was expressed as gram of oil emulsified by gram of protein.

Protein quality determination

The protein digestibility-corrected amino acid score (PDCAAS) was used to evaluate the protein quality of W-DPM and W-PcPI-Salt, using protein digestibility determined both in vivo and in vitro. W-PcPI-Salt was selected for PDCAAS analysis because of superior functionality compared with the alkali extracted PcPI samples, as will be discussed. Protein digestibility was determined in vivo as described by others (Bethesda, 1991; Hughes et al., 2011; Rutherfurd et al., 2015). All animal protocols were approved by the Institutional Animal Care and Use Committee. Briefly, male Sprague–Dawley rats ($n = 22$; 55–66 g initial body weight) were acclimated for three days by being fed ad libitum the AIN-93G purified diet. The rats were split into four groups and then offered diets containing either W-PcPI-SU-Salt (6 rats), W-DPM (6 rats), casein control (6 rats), or a protein-free control (4 rats). All protein containing diets were formulated to contain 12% protein. Each rat was offered 15 g/d of their respective diets for nine days. Feces were collected quantitatively from each rat daily for the last 5 days. At the end of the study, the 5-day fecal collection from each rat was composited, dried, and analyzed for nitrogen, and true digestibility was calculated as previously reported by Gilani and Sepehr (2003). Protein content ($N \times 6.25$) of fecal samples was determined by the Dumas method. In vitro protein digestibility was determined, in duplicate, using a commercial kit (K-PDCAAS, Megazyme, Bray, Ireland). Amino acid analysis was performed by Eurofins (Madison, WI, USA) using the methods of Henderson and Brooks (2010), Henderson et al. (2000), Schuster (1988), and the official methods of AOAC 988.15. PDCAAS was then calculated on the basis of WHO/FAO/UNU essential amino acid scoring pattern for children 6 months to 3 years (Joint FAO/WHO/UNU Expert Consultation, 2007) and as previously reported by Schaafsma (2000).

Statistical analysis

Analysis of variance (ANOVA) was performed using R (version 3.3.0). Significant differences ($p \leq 0.05$) among the means were determined by the Tukey–Kramer honest significant difference (HSD) mean comparison. A student's unpaired *t*-test was used to test for significant differences ($p \leq 0.05$) between the means of two different samples.

RESULTS AND DISCUSSION

Selection of optimal alkaline extraction conditions

The impacts of pH, duration, and number of solubilization, as well as the effect of degumming on the efficiency of protein extraction from W-DPM were evaluated (Table 1). When tested at pH 12 post degumming, increasing solubilization time from one 1- to 2-h did not significantly improve PcPI yield or purity. However, double (1 h) solubilization at pH 12 post degumming significantly increased the protein yield by ~9 percentage points compared to single (1 h) solubilization. Omitting the degumming step, on the other hand, resulted in a significant increase in protein yield (by ~14 percentage points) when extraction was performed following double solubilization for 1-h each at pH 12. Therefore, the impact of extraction pH was evaluated following double solubilization for 1-h without the degumming step. Extraction at pH 10 resulted in the highest protein purity but had the lowest protein yield due to significant residual protein in the discarded pellet. The protein yield observed at pH 10 was similar to that noted by Hojilla-Evangelista et al. (2015) for similar extraction conditions. Extraction at pH 12 resulted in the highest protein yield and an isolate with relatively high protein purity.

Highly alkaline conditions, however, may cause oxidation of residual phenolic compounds present in the W-DPM. Such oxidation causes protein–phenols interactions (Sosulski, 1979; Xu & Diosady, 2002), that, in turn, results in an undesirable dark brown to gray color (Blaicher et al., 1983; Lqari et al., 2002; Salgado et al., 2012). Additionally, protein–phenols interactions can be detrimental to the functionality of the protein (Balange & Benjakul, 2009; Rubino et al., 1996). Previous research showed that sodium sulfite, a reducing agent, reduced oxidation of phenolic compounds, resulting in lighter protein isolates from rapeseed, chickpea, sunflower, and lupin (Das Purkayastha & Mahanta, 2014; Rubin et al., 1990;

TABLE 1 Protein extraction purities (%) and yields (%) of the pennycress protein isolate (PcPI) and pellet fractions from alkaline extractions under different conditions.

Extraction conditions					PcPI		Discarded pellet[1]	
Degumming	# of solubilizations	Solubilization time (h)	pH	Sodium sulfite (%)	Protein purity (%)	Protein yield[2] (%)	Protein (%)	Protein residue[3] (%)
Yes	1	1	12	-	83.5^{bc*}	27.0^{ef}	$12.2^{b\dagger*}$	$18.2^{c\dagger*}$
Yes	1	2	12	-	80.6^{c}	28.1^{ef}	10.3^{c}	$16.0^{de\dagger}$
Yes	2	1	12	-	85.1^{abc}	$36.2^{cd\dagger}$	9.3^{cd}	14.1^{e}
Yes	2	2	12	-	83.0^{bc}	30.6^{de}	9.8^{cd}	13.6^{e}
No	2	1	10	-	90.5^{aA}	23.7^{fC}	17.0^{aA}	25.4^{aA}
No	2	1	11	-	86.9^{abB}	37.1^{cB}	9.7^{cdB}	13.9^{eC}
No	2	1	12	-	84.3^{abcCy}	$50.8^{abA1\wedge}$	8.8^{dBz}	$16.7^{cdBz\wedge}$
No	2	1	12	0.1	84.9^{abcy}	53.1^{ax}	8.8^{dz}	17.0^{cdz}
No	2	1	12	0.2	86.7^{abcx}	45.0^{by}	10.1^{cdy}	21.1^{by}
No	2	1	12	0.4	81.8^{bcz}	31.6^{cez}	12.7^{bx}	25.8^{ax}
SEM[4]					0.90	3.22	0.80	1.43

Note: $^{a-f}$Means ($n = 3$) in each column with different lowercase letters indicate significant differences across extraction treatments, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$); ^{A,B}Means ($n = 3$) with different capital letters indicate significant differences across different solubilization pHs, according to the Tukey–Kramer multiple means comparison ($p < 0.05$); $^{x-z}$Means ($n = 3$) with different x–z letters indicate significant differences across different concentrations of sodium sulfite used, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$). †Designates a significant difference between corresponding samples regarding single or double solubilization, as tested by the Student's two-sample unpaired *t*-test ($p < 0.05$). *Denotes a significant difference between corresponding samples regarding solubilization duration, as tested by the Student's two-sample unpaired *t*-test ($p < 0.05$). ^Denotes a significant difference between corresponding samples that either had or did not have a degumming step, as tested by the student's two-sample unpaired *t*-test ($p < 0.05$).
[1]Pellet discarded after alkaline solubilization.
[2]Protein Yield (%), the amount of protein extracted relative to the total amount of protein in the starting defatted pennycress meal.
[3]Protein residue (%), the amount of protein that remains in the pellet relative to the total amount of protein in the starting DPM.
[4]Standard error of the mean.

Sánchez-Vioque et al., 1999). Therefore, sodium sulfite at 0.1%, 0.2%, or 0.4% was added during alkaline solubilization at pH 12 (Table 1). At 0.2% and 0.4%, solubility at pH 12 was reduced as indicated by higher residual protein in the discarded pellet and lower protein yield compared with solubilization at pH 12 in the absence of sodium sulfite.

At an inclusion level of 0.1%, sodium sulfide had no significant effect on the protein yield. Higher amounts of sodium sulfite likely enhanced the formation of insoluble protein aggregates as observed in Figure 1a. SDS-PAGE showed that the PcPI produced using 0.2% and 0.4% sodium sulfite had pronounced smearing in the upper region of the lanes and banding in the wells (Figure 1a, lanes 4 and 5), indicative of the presence of high-molecular-weight polymers, and had reduced band intensity of individual protein bands between 37 and 50 kD compared to PcPI with zero and 0.1% sodium sulfite (Figure 1a, lanes 2 and 3, respectively). The observed smearing was resolved under reducing conditions and the band intensities of the protein subunits were similar in all samples (Figure 1a, lanes 6–9), indicating that polymerization/aggregation in the presence of 0.2% and 0.4% of sodium sulfite occurred via disulfide interchange. Protein aggregation induced by higher amounts of sodium sulfite may have a negative impact on protein functionality. Decreased protein functionality will outweigh the benefit of enhanced lightness observed with higher amounts of sodium sulfite. Nevertheless, inclusion of 0.1% sodium sulfite resulted in a significant increase in L^* (lightness; Table S1). Therefore, 0.1% sodium sulfite was chosen for alkaline extraction at pH 12, and the PcPI (W-PcPI-pH-S) produced was characterized alongside the isolate produced without sodium sulfite inclusion (W-PcPI-pH).

Selection of optimal salt extraction conditions

The impact of salt concentration (M NaCl), number of solubilizations, pH, and dialysis on the efficiency of protein extraction from W-DPM were evaluated (Table 2). At 0.5 M NaCl, pH ~6.8, double solubilization, compared with single solubilization, coupled with dialysis post ultrafiltration resulted in a significant increase in purity by ~16 percentage points, as well as modestly enhanced yield (Table 2). To further improve protein extraction yield, pH adjustments and higher salt concentrations were tested. Adjusting the extraction pH to 7 or 8 resulted in a significant decrease in yield, while increasing salt concentration did not significantly enhance protein yield. Therefore, double solubilization at 0.5 M NaCl followed by ultrafiltration and dialysis were the chosen parameters for the production of PcPI

from W-DPM (W-PcPI-Salt) and 0EA-DPM (0EA-PcPI-Salt).

The chosen salt extraction conditions resulted in similar protein yield and purity to that previously reported for pennycress protein extracted using 0.1 M NaCl followed by ultrafiltration without the use of dialysis (Hojilla-Evangelista et al., 2014). However, in a subsequent study by Hojilla-Evangelista et al. (2015), ultrafiltration alone was not effective in reducing the salt content resulting in a low protein purity (66%).

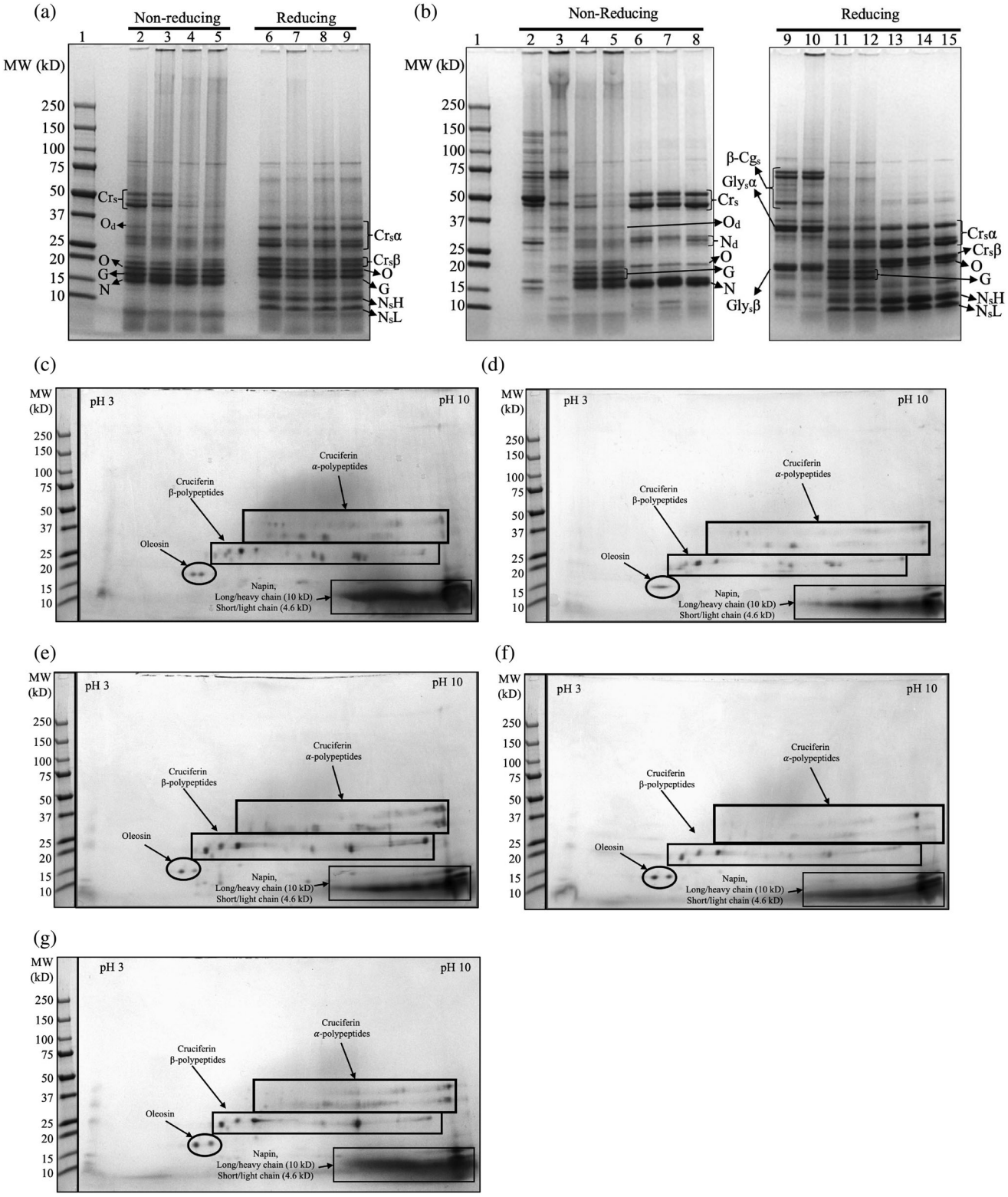

FIGURE 1 Legend on next page.

TABLE 2 Protein extraction purities (%) and yields (%) of the pennycress protein isolate (PcPI) and pellet fractions from salt extractions under different conditions.

Extraction conditions				PcPI		Discarded pellet[1]	
M NaCl	# of solubilizations	pH	Dialyzed	Protein purity (%)	Protein yield[2] (%)	Protein (%)	Protein residue[3] (%)
0.5	1	(∼6.3)	No	74.8^{d}	39.1^{b}	18.7^{a}	42.9^{a}
0.5	2	(∼6.3)	Yes	90.7^{bcBx}	42.6^{aAy}	17.6^{ax}	39.6^{abx}
0.5	2	7	Yes	96.1^{aA}	38.0^{bcB}	18.3^{a}	42.7^{a}
0.5	2	8	Yes	94.1^{abA}	36.5^{cB}	17.9^{a}	41.8^{a}
0.75	2	(∼6.3)	Yes	89.0^{cy}	43.3^{axy}	16.1^{aby}	38.2^{by}
1.0	2	(∼6.3)	Yes	88.9^{cy}	44.4^{ax}	15.5^{bz}	38.7^{bxy}
SEM[4]				3.06	1.31	0.52	0.85

Note: $^{a\text{-}d}$Means ($n = 3$) in each column with different lowercase letters indicate significant differences across extraction treatments according to the Tukey–Kramer multiple means comparison test ($p < 0.05$); $^{A\text{-}B}$Means ($n = 3$) with different capital letters indicate significant differences across different solubilization pH, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$); $^{x\text{-}z}$Means ($n = 3$) with different x-z letters indicate significant differences across different molarities of NaCl used, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).
[1]Pellet discarded after salt solubilization.
[2]Protein yield (%) represents the amount of protein extracted relative to the total amount of protein in the starting defatted pennycress meal.
[3]Protein residue (%) represents the amount of protein that remains in the pellet relative to the total amount of protein in the starting DPM.
[4]Standard error of the mean.

In this work, ultrafiltration alone was also ineffective in removing all excess salt from the extracted protein. Therefore, dialysis was added to reduce residual salt content. However, utilizing ultrafiltration coupled with diafiltration in the pilot plant was sufficient to reduce the salt content of W-PcPI-SU-Salt. Furthermore, salt extracted PcPI had a significantly higher L^* (lightness) value compared with both alkali extracted PcPI (Table S1). Therefore, this research proved that salt solubilization coupled with membrane filtration is scalable and effective in producing PcPI with low salt content and desirable light color. The relatively low protein yield is comparable to that of isolates from other oilseeds, such as camelina (Boyle et al., 2018) and rapeseed (Fetzer et al., 2018) produced by salt extraction.

Pennycress protein profile and differences in molecular weight distribution

Under nonreducing conditions, two cruciferin protein bands around the 50 kD mark were apparent in all PcPI samples, along with a prominent napin protein band around 15 kD (Figure 1b, lanes 4–8). Cruciferin, an 11S legumin-type globular protein, and napin, a 2S albumin-type protein, account for approximately 80%–85% of the total proteins in *Brassicaceae* seeds, including pennycress (Perera et al., 2016; Wanasundara, 2011). A cruciferin polypeptide consists of a heavy acidic subunit (α, 25–37 kD) and a light basic subunit (β, ∼20 kD) associated via disulfide linkage (Wanasundara et al., 2012). These subunits can be seen when the samples were run under reducing conditions (Figure 1b, lanes 11–15). Similarly, a napin polypeptide consists of two subunits linked via two disulfide bonds. While both subunits are basic, one is a heavy chain subunit (∼10 kD) and the other is a lighter chain subunit (∼4.5 kD). The multiple bands attributed to each of cruciferin and napin are considered genetic variants that vary slightly in amino acid composition and length (Wanasundara et al., 2012). A band at ∼20 kD visible under nonreducing conditions could be attributed to oleosin proteins (Li et al., 2002). Furthermore, glutelin-type protein bands around 15–20 kD (Li et al., 2014)

FIGURE 1 SDS-PAGE gel visualization of the protein profiles. (a) pH extracted pennycress protein isolates (PcPIs) without sodium sulfite (lanes 2 and 6) and 0.1% (lanes 3 and 7), 0.2% (lanes 4 and 8), and 0.4% (lanes 5 and 9) sodium sulfite under nonreducing (lanes 2–5) and reducing conditions (lanes 6–9). (b) Native soy protein isolate (nSPI; lanes 2 and 9), commercial soy protein isolate (cSPI; lanes 3 and 10), wild type pH extracted pennycress protein isolate (W-PcPI-pH; lanes 4 and 11), wild type pH extracted with 0.1% sodium sulfite pennycress protein isolate (W-PcPI-pH-S; lanes 5 and 12), wild type salt extracted pennycress protein isolate (W-PcPI-Salt; lanes 6 and 13), wild type scaled up salt extracted pennycress protein isolate (W-PcPI-SU-Salt; lanes 7 and 14), and zero erucic acid salt extracted pennycress protein isolates (0EA-PcPI-Salt; lanes 8 and 15) under nonreducing (lanes 2–8) and reducing (lanes 9–15) conditions. Cr_s: cruciferin subunits; O: oleosin; O_d: oleosin dimer; G: glutelin; N: napin; $Cr_s\alpha$: α- (acidic) subunits of cruciferin; $Cr_s\beta$: β- (basic) subunits of cruciferin; N_sH: heavy subunit of napin; N_sL: light subunit of napin; β-Cg_s: subunits of β-conglycinin; $Gly_s\alpha$: α- (acidic) subunits of glycinin; $Gly_s\beta$: β- (basic) subunits of glycinin. 2D gel electrophoresis (linear pH 3–10 range) visualization of (c) wild type pH extracted pennycress protein isolate (W-PcPI-pH); (d) wild type pH extracted with 0.1% sodium sulfite pennycress protein isolate (W-PcPI-pH-S); (e) wild type salt extracted pennycress protein isolate (W-PcPI-Salt); (f) wild type scaled up salt extracted pennycress protein isolate (W-PcPI-SU-Salt); and (g) zero erucic acid salt extracted pennycress protein isolate (0EA-PcPI-Salt).

were observed in W-PcPI-pH and W-PcPI-pH-S (Figure 1b, lanes 4 and 5), which were not apparent in the salt extracted samples (Figure 1b, lanes 6, 7, and 8). Glutelin-type proteins are soluble under high alkalinity, yet insoluble in a salt solution (Li et al., 2014). Protein bands observed at ~30 kD in all PcPI samples under nonreducing conditions could be attributed to napin dimers (Perera et al., 2016). Oleosins also form dimers (Li et al., 2002), which are only observed at ~35–37 kD in the alkali extracted PcPI samples (Figure 1b, lanes 4 and 5).

Compared with salt extracted samples, both W-PcPI-pH and W-PcPI-pH-S had considerable smearing in the upper region of the gel, which indicated the presence of high molecular weight polymers that formed during extraction under high alkalinity (Figure 1b, compare lanes 4 and 5 to lanes 6–8). The smearing was less prevalent under reducing conditions, indicating that disulfide interchange was induced during alkaline extraction and more so in the presence of sodium sulfite (Figure 1b, compare lanes 4 and 5 to lanes 11 and 12). Hojilla-Evangelista et al. (2014) observed a relatively more noticeable protein aggregation in alkali extracted PcPI compared with salt extracted PcPI even under reducing conditions, indicating excessive covalent aggregation attributed to alkaline extraction at 50°C. In the current study, alkaline extraction at room temperature resulted in higher PcPI yields than what was previously reported (Hojilla-Evangelista et al., 2014, 2015) with relatively less covalent aggregation, which may contribute to enhanced protein functionality.

The protein profile of salt extracted PcPI was not majorly impacted by scale-up production in the pilot plant, despite the additional heat treatments during pasteurization and spray drying (Figure 1b, lanes 6 and 7). Only a slight difference in the band intensity of the napin dimer and a faint presence of dissociated napin subunits were observed in W-PcPI-SU-Salt compared with W-PcPI-Salt. This observation could be attributed to a mild disulfide interchange induced by the heat treatments (pasteurization and spray drying; Hansen et al., 2022). Similarly, no major differences in protein profile were noted as a result of breeding, where W-PcPI-Salt and 0EA-PcPI-Salt had similar protein bands and intensity (Figure 1b, lanes 6, 8, 13, and 15).

As reference samples, nSPI and cSPI were run on the same gel to monitor differences in globulin proteins. Both nSPI and cSPI had globulin proteins (β-conglycinin and glycinin) of markedly higher molecular weight than those of the PcPI samples (Figure 1b, lanes 2 and 9 and lanes 3 and 10, respectively, compared to lanes 4–8 and 11–15). Compared to nSPI, the sample produced in the lab under mild and nonthermal conditions, cSPI showed higher degree of polymerization, attributed to thermal processing. Differences in molecular weight distribution and presence of higher molecular weight polymers will impact functional properties (Damodaran, 2017).

2D gel electrophoresis of PcPI samples showed that napin's heavy (~10 kD) and light (~4.5 kD) subunits extended across the alkaline region of the gel, with heightened intensity closer to pH 10 (Figure 1c–g). This observation is in accordance with the reported pI of napin subunits ranging from pH 10.25 to 12.16 (Crouch et al., 1983). Boyle et al. (2018) similarly observed camelina napin subunits along the alkaline region of the 2D gel. Cruciferin α- and β- subunits extended horizontally across the middle region of the gel corresponding to a wide pI range similar to the previously reported pH 6.7–8.8 and pH 5.9–9.5, respectively, for canola cruciferin subunits (Nietzel et al., 2013). The numerous cruciferin spots are indicative of several genetic variants having different amino acid composition (Wanasundara, 2011). Two oleosin spots in rapeseed protein were observed at ~19 kD toward the more acidic pH range, which is consistent with the reported pI range, ~pH 5.5–6.6 (Tzen et al., 1997).

Molecular weight distribution of soluble proteins in the different PcPI samples as determined by SE-HPLC is presented in Table 3. When samples were solubilized in phosphate buffer there was no disruption of noncovalent interactions and disulfide linkages. Observed chromatographic peaks represented soluble aggregates (~900–2600 kD), hexameric cruiferin (~400 kD), oleosin dimers (~40 kD), and monomeric napin (~17 kD). For alkali extracted samples, relative abundance of soluble aggregates and cruciferin significantly increased upon solubilization in buffer containing SDS, compared with salt extracted samples (Table 3). The high alkalinity during the extraction most likely contributed to protein denaturation and consequent polymerization via hydrophobic interactions, resulting in the formation of insoluble aggregates that were filtered out prior to sample injection. However, in the presence of SDS, some hydrophobic interactions were disrupted contributing to a relatively higher abundance of soluble aggregates and hexameric cruciferin. The higher relative abundance of hexameric cruciferin upon solubilization in SDS indicated that cruciferin formed aggregates via noncovalent interactions. When solubilized in buffer containing both SDS and BME, a slight decrease in hexameric cruciferin was noted and attributed to partial dissociation of cruciferin into the monomeric form. Additionally, insoluble aggregates formed by noncovalent interactions and disulfide linkages were solubilized by disrupting both noncovalent interactions and disulfide linkages. Results indicated a combined effect of partial dissociation of insoluble aggregates into soluble aggregates coupled with dissociation of soluble aggregates into hexameric cruciferin and further into monomers.

Under mild salt extraction conditions, the hexameric cruciferin had relatively higher abundance in PcPI samples across different solubilizing buffers. This observation complemented that of the SDS-PAGE, where minimal if any smearing (polymerization) was noted in

TABLE 3 Molecular weight and relative abundance (%) of soluble aggregates, cruciferin, glutelin, and napin in pennycress protein isolates from wild-type and zero erucic acid pennycress lines as determined by size-exclusion high performance liquid chromatography (SE-HPLC) using three different sample buffers.

Relative abundance (%) of protein fractions[2]												
	Phosphate buffer[3]				Phosphate buffer (0.1% SDS)[4]				Phosphate buffer (0.1% SDS + 2.5% BME)[5]			
Samples[1]	Soluble aggregates (900–2600 kD)	Cruciferin (~400 kD)	Oleosin dimers (~40 kD)	Napin (~17 kD)	Soluble aggregates	Cruciferin	Oleosin dimers	Napin	Soluble aggregates	Cruciferin	Oleosin dimers	Napin
W-PcPI-pH	10.16^{aC}	13.01^{cB}	24.35^{aA}	19.10^{bA}	17.99^{bB}	19.35^{cA}	9.42^{bB}	12.63^{bB}	24.05^{bA}	12.16^{bB}	10.90^{aB}	*
W-PcPI-pH-S	7.96^{cC}	12.08^{cC}	22.71^{aA}	17.28^{cA}	26.60^{aB}	16.28^{dA}	12.82^{aB}	13.97^{aB}	27.61^{aA}	11.89^{bB}	8.58^{bC}	*
W-PcPI-Salt	9.03^{bB}	54.10^{aB}	2.75^{b}	17.77^{bcA}	11.43^{cA}	62.46^{bA}	*	4.97^{dC}	11.52^{cA}	47.00^{aC}	*	6.40^{aB}
W-PcPI-SU-Salt	6.60^{dB}	51.12^{bB}	1.98^{b}	22.03^{aA}	5.82^{dC}	64.65^{aA}	*	8.41^{cB}	7.43^{eA}	46.79^{aC}	*	6.06^{bC}
0EA-PcPI-Salt	5.80^{eC}	55.84^{aB}	*	17.91^{bcA}	7.45^{dB}	64.75^{aA}	1.50^{c}	4.34^{eB}	8.46^{dA}	47.57^{aC}	*	2.92^{cC}
SEM[6]	0.79	10.11	6.12	0.86	3.81	11.32	3.35	1.95	4.18	8.60	1.16	0.86

Note: $^{a–c}$Means ($n = 3$) in each column with different lowercase letters indicate significant differences across the samples, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$); $^{A–C}$Means ($n = 3$) in each row with different uppercase letters indicate significant differences within each protein fraction across solubilization buffer type for each sample, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).

[1]Samples include pennycress protein isolates extracted from wild-type defatted pennycress meal following alkaline extraction (W-PcPI-pH), alkaline extraction with 0.1% sodium sulfite (W-PcPI-pH-S), bench-top salt extraction (W-PcPI-Salt), scaled up salt extraction (W-PcPI-SU-Salt), and pennycress protein isolate extracted from zero erucic acid defatted pennycress meal following salt extraction (0EA-PcPI-Salt).

[2]Relative abundance (%) is the area of a specific peak divided by the total peak area.

[3]Samples were dissolved in 0.05 M sodium phosphate buffer, pH 7, 0.1 M NaCl.

[4]Samples were dissolved in 0.05 M sodium phosphate buffer, pH 7, 0.1 M NaCl with 0.1% SDS.

[5]Samples were dissolved in 0.05 M sodium phosphate buffer, pH 7, 0.1 M NaCl with the 0.1% SDS and 2.5% BME.

[6]Standard error of the mean.

*Denotes no apparent peak in this molecular weight range.

the upper region of the gel (Figure 1b). A decrease in the relative abundance of hexameric cruciferin was observed when the salt extracted PcPI samples were solubilized in the presence of BME. This observation was attributed to the partial dissociation into acidic and basic subunits.

Differences in the relative abundance of napin were minimal across the samples, with a significant reduction noted when the samples were dissolved in a buffer containing SDS and BME. This reduction and, in some cases, disappearance of the monomeric napin could be mostly attributed to the dissociation into the small molecular weight heavy and light subunits. Oleosin dimers, on the other hand, were either absent or present in low amounts in salt extracted PcPI compared to alkali extracted PcPI, as was observed by SDS-PAGE (Figure 1b). Therefore, it was concluded that alkaline conditions catalyzed oleosin dimer formation. The decrease in oleosin during dissolution with SDS or SDS and BME was attributed to the dissociation of the dimers into monomers.

Differences in protein denaturation state among the PcPI samples

Two endothermic peaks were observed for most PcPI samples (Table 4). The first endothermic peak with a denaturation temperature (T_d) of ~83–88°C was attributed to cruciferin, while the second endothermic peak with a T_d of ~101–107°C corresponded to napin (Wu & Muir, 2008). The high alkalinity employed during the production of W-PcPI-pH and W-PcPI-pH-S resulted in almost complete denaturation of cruciferin as indicated by its very low enthalpy compared with that of cruciferin in the salt extracted PcPI samples. This observed denaturation state of cruciferin in alkali extracted PcPI contributed to the formation of large molecular weight aggregates (Figure 1b).

The cruciferin in W-PcPI-SU-Salt had significantly lower enthalpy than that of W-PcPI-Salt, most likely due to the exposure to heat during pasteurization and spray drying that could have resulted in partial unfolding. Similarly, Elmore et al. (2007) observed that pasteurization and spray drying of SPI caused partial denaturation compared to lyophilized SPI. For reference purposes, both nSPI and cSPI were evaluated for their protein denaturation state. While two distinct endothermic peaks corresponding to β-conglycinin and glycinin were observed for nSPI, none was detected for cSPI, further confirming the impact of thermal processing on the denaturation state of the protein. This observation also explained the excessive polymerization noted for cSPI compared to nSPI (Figure 1b).

Cruciferin in 0EA-PcPI-Salt had a significantly lower enthalpy than that in W-PcPI-Salt. Given that the salt extraction conditions were the same for both isolates, the observed difference in denaturation enthalpy could be attributed to genetic differences in the cruciferin variants in the wild type compared with the 0EA accession. Similarly, Withana-Gamage et al. (2013) observed that genetic variants of *Arabidopsis* cruciferin resulted in differences in both T_d and enthalpy. On the other hand, the napin endothermic peak was generally a small and not well-defined peak, resulting in minor differences among samples. Boyle et al. (2018) also observed small peak for napin in camelina protein isolate, whereas Wanasundara et al. (2022) did not observe a napin endothermic peak. Differences in the extent of protein denaturation will impact other structural properties that consequently influence functionality.

Difference in surface properties among PcPI samples

Upon denaturation, hydrophobic residues in the interior moiety of the protein are exposed, thus increasing surface hydrophobicity (Foegeding & Davis, 2011; Kato & Nakai, 1980). As expected, cSPI had a significantly higher surface hydrophobicity than nSPI (Table 4), which supported the extent of denaturation indicated by the lack of endothermic peaks. Similarly, the protein denaturation observed for alkali extracted PcPI compared to salt extracted PcPI contributed to a significantly higher surface hydrophobicity. Additionally, thermal processing during the production of W-PcPI-SU-Salt resulted in significantly higher surface hydrophobicity compared to W-PcPI-Salt. On the other hand, the higher surface hydrophobicity of 0EA-PcPI-Salt compared to W-PcPI-Salt, could be attributed to differences in amino acid composition and sequence due to possible genetic variance.

W-PcPI-pH-S had a greater surface hydrophobicity compared to W-PcPI-pH. The presence of sodium sulfite induced disulfide rearrangement and interchange that could have contributed to conformational changes that resulted in increased surface hydrophobicity. Similarly, sunflower protein isolate extracted under alkaline conditions with sodium sulfite had greater surface hydrophobicity than that of alkali extracted isolate without sodium sulfite (Salgado et al., 2011).

Along with surface hydrophobicity, surface charge has a direct bearing on protein functionality, including solubility, gelation, and emulsification properties. While there were some differences in the surface charge (zeta potential, ζ) among samples, all isolates carried a net negative surface charge, as would be expected because measurements were carried out at pH 7, which was above the pI of the protein isolates (Table 4). The surface charge of alkali extracted PcPI was higher than the reported value for alkali extracted canola protein (~−23 mV; Kim et al., 2016), and significantly higher than that of salt extracted PcPI, which

TABLE 4 Denaturation temperatures and enthalpy, surface hydrophobicity, surface charge, and secondary structure of pennycress protein isolates and native and commercial soy protein isolates (nSPI and cSPI, respectively).

	Denaturation temperature and enthalpy				Surface properties		Secondary structure			
					Surface hydrophobicity	Surface charge	Relative percentage (%)			
Samples[1]	Denaturation temperature (T_d, °C)	Enthalpy (ΔH, J g^{-1})	Denaturation temperature (T_d, °C)	Enthalpy (ΔH, J g^{-1})	RFI	mV	α-Helix	β-Sheet	β-Turn	Random Coil
	Cruciferin		*Napin*							
W-PcPI-pH	83.43^{b}	0.33^{d}	101.51^{c}	0.45^{c}	9462.57^{b}	−35.14^{c}	28.25^{a}	35.74^{b}	24.78^{ab}	11.23^{a}
W-PcPI pH-S	83.91^{ab}	0.05^{d}	105.05^{ab}	1.40^{b}	11830.67^{a}	−32.90^{c}	27.26^{a}	42.69^{a}	23.40^{ab}	5.31^{c}
W-PcPI-Salt	85.92^{a}	7.28^{a}	107.66^{a}	0.16^{c}	7164.60^{c}	−10.09^{a}	26.91^{a}	45.01^{a}	21.51^{b}	6.58^{bc}
W-PcPI-SU-Salt	87.79^{a}	5.80^{b}	102.73^{bc}	0.03^{c}	10027.23^{b}	−7.95^{a}	19.62^{b}	42.56^{a}	29.49^{a}	8.33^{abc}
0EA-PcPI-Salt	86.54^{a}	6.23^{b}	*	*	9217.57^{b}	−13.60^{b}	21.75^{b}	44.37^{a}	25.42^{ab}	8.46^{ab}
	β-conglycinin		*Glycinin*							
nSPI	72.97^{c}	1.51^{c}	90.89^{d}	7.1^{a}	5891.17^{d}	−42.38^{d}	NA	NA	NA	NA
cSPI	*	*	*	*	10056.83^{b}	−41.90^{d}	NA	NA	NA	NA
SEM[2]	2.20	1.33	2.96	1.34	746.7	5.74	0.89	2.68	1.27	2.25

Note: NA indicates not analyzed; $^{a-d}$Means ($n = 3$) in each column with different lowercase letters indicate significant differences among samples within each structural analyses, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).

[1]Samples include pennycress protein isolates extracted from wild-type defatted pennycress meal following alkaline extraction (W-PcPI-pH), alkaline extraction with 0.1% sodium sulfite (W-PcPI-pH-S), bench-top salt extraction (W-PcPI-Salt), scaled up salt extraction (W-PcPI-SU-Salt), pennycress protein isolate extracted from zero erucic acid defatted pennycress meal following salt extraction (0EA-PcPI-Salt), native soy protein isolate (nSPI), and commercial soy protein isolate (cSPI);

[2]Standard error of the mean.

*Denotes no endothermic peak observed.

was slightly lower than what was reported for salt extracted canola protein (∼−15 and −20 mV; Chang et al., 2015; Kim et al., 2016). However, when the surface charge of salt extracted canola protein isolate was measured in 0.1 M NaCl, there was less overall surface charge compared to when measured in water (Kim et al., 2016). Therefore, the lower surface charge of salt extracted PcPI samples observed in this study could be attributed to their relatively higher ash content (6.1%–6.8%), where salt could have shielded charges on the surface of the protein.

The observed difference in surface charge between alkali extracted PcPI and salt extracted PcPI samples could be partially attributed to the residual salt content, as well as differences in protein composition. During the purification of alkali extracted PcPI, a pI of 5 was used, which would be selective for proteins that would have a net negative charge above this pI. In contrast, purification of salt extracted PcPI was not pH dependent; therefore, the isolate would have different protein subunits with varying pI values, contributing to different net negative charges at pH 7. The slightly higher net charge of 0EA-PcPI-Salt compared with the other salt extracted PcPI samples could be attributed to slight differences in amino acid composition (Table S2). In comparison, the surface charge of SPI samples was found to be significantly higher than the alkali extracted PcPI samples, mostly because soy protein has a more acidic pI (4.5 on average) than pennycress protein.

Differences in protein secondary structures among PcPI samples

Differences in protein secondary structures may indicate compositional variations as well as possible denaturation at the secondary level. Differences in the α-helix, β-sheet, β-turn, and random coil contents in PcPI samples (Table 4) may contribute to variation in functional properties. The relative ratio of cruciferin to napin extracted under different conditions may impact the secondary structure distribution. Napin contains a larger proportion of α-helix (∼40%–46%) than β-sheet (∼12%), whereas cruciferin contains more β-sheet (∼50%) than α-helix (∼10%; Schwenke, 1994; Zirwer et al., 1985). The significantly higher β-sheet in salt extracted PcPI samples compared with the W-PcPI-pH sample could be partially attributed to a higher proportion of cruciferin. However, this inference needs to be further validated by quantitative measures. On the other hand, the lower α-helix to β-sheet ratio in W-PcPI-SU-Salt compared with W-PcPI-Salt could be attributed to protein denaturation due to thermal processing during the pilot scale production. Yu et al. (2005) observed that golden flax seed protein had less α-helix and more β-sheet upon roasting. An increase in β-sheet structures may have a positive impact on functionality as it has been correlated with enhanced gelling and emulsification properties (Cao & Mezzenga, 2019; Przybycien & Bailey, 1991). W-PcPI-pH had the lowest proportion of β-sheet compared with all other samples and had the most random coil structures. This observation indicated that a potential conversion of β-sheets to unordered structures had occurred due to the extensive denaturing effects of high alkalinity during the extraction.

Protein solubility

At pH 3.4 under both nonheated and heated conditions, W-PcPI-Salt and W-PcPI-SU-Salt were highly soluble, outperforming cSPI (Table 5). This relatively high solubility of these PcPI samples may be attributed to the intact globular structures, allowing hydrophobic residues to remain within the interior moiety and the relatively hydrophilic residues on the surface to associate with water (Damodaran, 2017). The similar solubility of W-PcPI-SU-Salt to that of W-PcPI-Salt indicated that the previously noted partial denaturation upon scale up, and the slightly higher surface hydrophobicity (Table 4) did not negatively impact solubility under acidic conditions. The SDS-PAGE (Figure 1b, lanes 6–7, and 13–14) and molecular weight profile (Table 3) confirmed that minimal, if any, polymerization occurred upon scale up. Additionally, the tested acidic pH is potentially farther away from the average pI of pennycress protein. The relatively low solubility of cSPI, on the other hand, was not only due to the tested acidic pH being close to the pI of soy protein, but also due to the extent of protein denaturation, the relatively higher surface hydrophobicity (Table 4), and the presence of higher molecular weight aggregates (Figure 1b, lane 3).

The lower solubility of alkali extracted PcPI compared with salt extracted counterparts at both pH 3.4 and pH 7 is attributed to significant denaturation (Table 4) and excessive polymerization (Figure 1b, lanes 4 and 5, and Table 3), despite having higher surface charge at pH 7. The similar solubility of W-PcPI-pH-S to that of W-PcPI-pH indicated that the slight polymerization observed (Figure 1b, compare lanes 4 and 5) did not impact the interactions with water. At pH 7, both nSPI and cSPI were much more soluble than all of the PcPI samples. This was mostly attributed to the low solubility of pennycress protein at neutral pH, which is relatively close to its average pI. Additionally, both nSPI and cSPI carried the highest net negative charge at pH 7 (Table 4).

Upon heating globular protein structures potentially unfold, exposing hydrophobic residues, decreasing protein–water interactions, and thus may result in reduced solubility (Damodaran, 2017). This theory proved true for all salt extracted samples upon heating at pH 7. This observation was attributed to having the least surface charge at pH 7 among the samples, as a

TABLE 5 Protein solubility, water holding capacity (WHC), gel strength and emulsification capacity (EC) of pennycress protein isolates, native and commercial soy protein isolate (nSPI and cSPI, respectively).

	Solubility (%)						
	pH 3.4		pH 7		WHC	Gel strength	EC
Samples[1]	Non-heated[2]	Heated[3]	Non-heated	Heated	%	Strength (N)	g oil/g protein
W-PcPI-pH	31.40^{c}	43.85^{d}*	27.44^{d}	28.32^{e}	98.36^{a}	7.60^{d}	768.8^{d}
W-PcPI-pH-S	24.71^{d}	40.87^{d}*	30.76^{d}	30.67^{de}	98.38^{a}	4.35^{e}	706.8^{d}
W-PcPI-Salt	71.30^{a}	78.59^{b}*	62.82^{c}*	34.27^{d}	74.47^{c}	9.45^{c}	1739.1^{a}
W-PcPI-SU-Salt	68.58^{ab}	64.00^{c}	64.65^{c}*	43.33^{c}	81.84^{b}	11.78^{b}	1674.0^{a}
0EA-PcPI-Salt	34.74^{c}	35.14^{d}	73.92^{b}*	45.93^{c}	71.28^{c}	9.34^{c}	1748.4^{a}
nSPI	65.42^{b}	88.87^{a}*	95.47^{a}	91.61^{a}	100.01^{a}	17.18^{a}	1295.8^{b}
cSPI	27.85^{cd}	24.05^{e}	79.83^{b}	84.60^{b}	99.98^{a}	11.14^{b}	1102.1^{c}
SEM[4]	7.95	9.06	9.46	9.84	4.86	1.50	169.48

Note: $^{a\text{-}d}$Means ($n = 3$) in each column with different lowercase letters indicate significant differences among samples, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).
[1]Samples include pennycress protein isolates extracted from wild-type defatted pennycress meal following alkaline extraction (W-PcPI-pH), alkaline extraction with 0.1% sodium sulfite (W-PcPI-pH-S), bench-top salt extraction (W-PcPI-Salt), scaled up salt extraction (W-PcPI-SU-Salt), pennycress protein isolate extracted from zero erucic acid defatted pennycress meal following salt extraction (0EA-PcPI-Salt), native soy protein isolate (nSPI), and commercial soy protein isolate (cSPI).
[2]Non-heated designates samples were prepared at ambient conditions.
[3]Heated designates that samples were heated in a water bath at 80°C for 30 min.
[4]Standard error of the mean.
*Denotes a significant difference between non-heated and heat samples, as tested by the Student's two-sample unpaired *t*-test ($p < 0.05$).

consequence of the relatively higher ash content. The low surface charge coupled with protein unfolding upon heating would result in reduced solubility. On the other hand, a significant increase in solubility at pH 3.4 was noted upon heating of W-PcPI-Salt, W-PcPI-pH, W-PcPI-pH-S, and nSPI, which might be attributed to enhanced thermal kinetics and interactions with water, assuming a potentially higher net positive charge. With the higher positive charge, the protein will have higher thermal stability coupled with enhanced thermal kinetics, thus contributing to higher solubility at pH 3.4 under heated conditions.

Compared to W-PcPI-Salt and W-PcPI-SU-Salt, 0EA-PcPI-Salt had significantly lower solubility at pH 3.4 under both nonheated and heated conditions. The lower solubility of 0EA-PcPI-Salt is likely due to a higher surface charge at pH 7 (Table 4) compared with the wild-type salt extracted counterparts, which may indicate a comparatively lower average pI that moved closer to pH 3.4. A lower pI may be confirmed by the higher solubility of 0EA-PcPI-Salt at pH 7 compared to W-PcPI-Salt and W-PcPI-SU-Salt under non-heating conditions (Table 5), which was nullified upon heating. When a protein is heated closer to the isoelectric point, proteins will more readily denature, exposing hydrophobic residues and off-setting the hydrophilic/hydrophobic balance, thus decreasing solubility (Damodaran, 2017). These observed differences could be attributed to variations in amino acid profiles (Table S2), and the configuration of the protein due to potential differences in amino acid sequences between the two pennycress lines. This assumption, however, requires further evaluation using proteomics. In any case, results indicated that breeding could lead to differences in protein configuration, subsequently altering critical functional properties.

Solubility of W-PcPI-Salt and W-PcPI-pH under nonheated conditions at pH 7 was similar to what was previously reported for salt and alkali extracted PcPI (Hojilla-Evangelista et al., 2014). The current research, however, demonstrated beverage applicability by testing solubility under heated conditions to demonstrate how PcPI solubility could change with processing. Additionally, this work illustrated that salt extracted PcPI samples may show inferior solubility in neutral beverage systems compared to SPI, but when produced under industrially feasible conditions, it may readily replace SPI in acidic beverage systems.

Water holding capacity and gelation

Protein gels with high WHC have been correlated with greater juiciness and tenderness in food applications, such as meat analogues. nSPI, cSPI, W-PcPI-pH, and W-PcPI-pH-S had almost complete water retention, as indicated by their high WHC values compared to salt extracted PcPI samples (Table 5). However, salt extracted PcPI samples had lower WHC compared to alkali extracted PcPI, likely due to lower surface charge (Table 4) that resulted in lower protein–water interactions and ability to entrap water. Furthermore, residual salt in said samples might have led to gels being formed primarily by protein–protein interactions to have larger pores, reducing WHC. Hemp and whey protein gels formed at higher salt concentrations were reported to have large pores and water loss

(Chantrapornchai & McClements, 2002; Dapčević-Hadnađev et al., 2018). However, at higher ionic strengths, protein charge is shielded, decreasing electrostatic repulsion, enhancing protein–protein interactions, and thus forming stronger gels (Chantrapornchai & McClements, 2002).

Globular protein structures are favorable for solubility; however, the ability of proteins to aggregate and form a strong gel is dependent on a balance of attractive and repulsive (electrostatic) interactions (Papalamprou et al., 2009; Zheng et al., 1993). nSPI gels had the highest strength (Table 5), likely due to intact globular proteins that are higher in molecular weight compared to proteins in PcPI (Figure 1b). Larger molecular weight proteins are correlated positively with enhanced gel strength as they have more reactive residues available to form extensive gel networks compared to small molecular weight proteins (Kinsella, 1976). Despite containing higher molecular weight proteins (Figure 1b), gels formed with cSPI had lower strength than that of nSPI gels, likely due to cSPI being highly denatured (Table 4) and excessively polymerized (Figure 1b). Highly denatured and aggregated proteins, as in cSPI and alkali extracted PcPI samples (Table 4), form less ordered, weak gel networks due to random aggregation. Alternatively, intact globular proteins, as in nSPI and salt extracted PcPI samples (Table 4), denature upon controlled heating and form ordered, and relatively stronger gels (Hermansson, 1979; Tombs, 1974).

Furthermore, salt extracted PcPI had a relatively higher ratio of cruciferin to napin compared to alkali extracted PcPI (Figure 1b and Table 3). Napin is more heat stable than cruciferin, with a T_d of ~104°C (Table 4), has lower molecular weight, and more soluble; therefore, napin might have less protein–protein interactions upon heating at 95°C, potentially contributing to weaker gels (Campbell et al., 2016; Wu & Muir, 2008). On the other hand, cruciferin, which had a T_d of ~85°C, would more readily denature under the tested heating conditions to form stronger, more cohesive gel networks, as seen for the gels formed with salt extracted PcPI samples. W-PcPI-SU-Salt gels were the strongest among PcPI samples, and comparable to cSPI, which is attributed to the observed partial denaturation (Table 4) that enhanced protein–protein interactions upon further heating. Finally, variety had no apparent effect on gelation, given that W-PcPI-Salt and 0EA-PcPI-Salt gel strengths were similar. These results are promising for the potential use of salt extracted PcPI as partial or full replacement for cSPI in gel applications.

Emulsification capacity

EC requires proteins to have a relatively flexible structure and a good hydrophilic/hydrophobic balance to interact with both water and oil phases (Damodaran, 2017). This research is the first to report not only on thermal gelation but also on the EC for PcPI. Salt extracted PcPI had significantly higher EC than alkali extracted PcPI and was superior to both SPI samples (Table 5). Previous research reported that as extraction pH increased, the EC of canola protein isolates decreased (Pedroche et al., 2004). Therefore, extracting PcPI at lower alkalinity may be advantageous to improve functionality. Furthermore, the highly alkaline conditions employed during extraction and consequent protein denaturation and polymerization resulted in poor hydrophilic/hydrophobic balance as indicated by higher surface hydrophobicity (Table 4) and lower solubility (Table 5) of the alkali extracted PcPI samples, and hence contributed to poor EC. However, it must be noted that extracting pennycress protein at lower alkalinity will drastically reduce the protein yield.

Despite having high surface hydrophobicity, cSPI maintained high EC likely due to adequate hydrophilic/hydrophobic balance as indicated by higher solubility (Table 5) and surface charge at pH 7 (Table 4). The high EC of salt extracted PcPI also indicated favorable balance of hydrophilic/hydrophobic surface residues, as well as favorable flexibility and size of the intact globular structure. A relatively small globulin protein will have a fast migration to the interface. Salt extracted PcPI samples had higher EC than that reported for salt extracted camelina protein and salt extracted pea protein isolate (Boyle et al., 2018; Hansen et al., 2022). Hence, these results indicate that salt extracted PcPI can be superior to SPI and other plant proteins in food applications that rely on emulsion formation. Furthermore, results confirm the feasibility of producing highly functional salt extracted PcPI, since scaling up did not significantly impact the EC. Similarly, breeding for 0-EA content did not negatively impact the EC.

Protein quality

Based on the comparison of amino acid profile of DPM and W-PcPI-Salt to the reference amino acid pattern, lysine was determined as the limiting amino acid in pennycress (Table S2; Joint FAO/WHO/UNU Expert Consultation). The amino acid composition was similar to that reported by Hojilla-Evangelista et al. (2015). In vivo protein digestibility, on the other hand, was significantly greater in W-PcPI-Salt than in the DPM (Table 6), which may be due to protein compositional differences or the absence of glucosinolate content. Pennycress seeds inherently contain the glucosinolate sinigrin, which increases in concentration upon oil removal to produce DPM, and is reduced to negligible amounts during protein extraction and isolation (data not shown), as was also reported by Hojilla-Evangelista et al. (2015). Andersen et al. (2010) found that

TABLE 6 In vivo and in vitro protein digestibility and protein digestibility-corrected amino acid score (PDCAAS) of casein, wild-type defatted pennycress meal (W-DPM), and salt extracted pennycress protein isolate (W-PcPI-Salt).

Sample	In vivo protein digestibility score	In vitro protein digestibility score	In vivo PDCAAS	In vitro PDCAAS
Casein	0.95[a]	0.99[b]	1.0[a][1]	1.0[a][1]
W-DPM	0.71[b]	0.90[c]	0.65[c]	0.81[c]
W-PcPI-Salt	0.90[a]	1.11[a]	0.72[b]	0.87[b]
SEM[2]	0.08	0.09	0.14	0.07

Note: [a-c]Means ($n = 3$ or 6) in each column with different lowercase letters indicate significant differences among samples, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).
[1]Values exceeded 1.0. By convention, value was truncated to 1.0.
[2]Standard error of the mean.

glucosinolates differ in the degree to which they reduce the biological value of casein, but that sinigrin was one of the most potent. Biological value measures both protein digestibility and protein retention. Although protein digestibility differed between the in vivo and in vitro tests, the difference in protein digestibility in DPM relative to W-PcPI-Salt was similar, suggesting that the reduction in biological value by sinigrin found by Andersen et al. (2010) was due to a reduction in protein digestibility. In vitro protein digestibility was greater for both DPM and W-PcPI-Salt than in vivo digestibility, however the pattern was similar with W-PcPI-Salt higher in vivo digestibility than DPM.

The in vivo PDCAAS of DPM is high relative to other plant sources, namely beans, lentils, and peas, which have PDCAAS values in the range of 0.54–0.67 (Nosworthy & House, 2017). This high PDCAAS is largely due to the high amino acid score of DPM, which is greater than almost all cereals and many legumes (Young & Pellett, 1994). Although the amino acid score of W-PcPI-Salt was lower than DPM, the greater in vivo digestibility resulted in a significantly greater PDCAAS value for W-PcPI-Salt. Comparisons of the digestibility of the same plant protein as a concentrate and as an isolate seem to be lacking. However, protein in whole cowpea has been found to be less digestible than the protein from cowpea protein isolate in hamsters (Frota et al., 2017). The in vitro PDCAAS of both DPM and W-PcPI-Salt was significantly greater than the in vivo PDCAAS values, due to the greater in vitro protein digestibility. Alternatively, the in vivo and in vitro protein digestibility of casein was quite similar. The reason for the lack of correspondence between the in vivo and in vitro protein digestibility of pennycress protein is unclear. Further comparative studies are needed to evaluate the predictability of the in vitro digestibility assay. In any case, these results are unique and promising given that the PDCAAS of PcPI, while lower than that of SPI (0.95–0.99), is similar to other plant proteins, and in some cases better than others, such as gluten protein that has a PDCAAS of 0.42 (Hughes et al., 2011; Nosworthy et al., 2022).

CONCLUSIONS

For the first time, alkaline extraction conditions to produce PcPI were optimized for efficiency in terms of protein yield and purity. In addition, salt extraction, following mild conditions, was successfully scaled up to produce PcPI with lighter color, preserved structural properties, and better functionality than the alkali extracted counterpart. The partial denaturation and mild polymerization that occurred during process scale up of PcPI enhanced the hydrophilic/hydrophobic surface balance, which led to superior WHC and gelation. Furthermore, this research served as a benchmark for breeding efforts to help expedite the commercialization of pennycress as a viable source of plant protein. Specifically, this work showed that the protein isolated from the zero erucic acid accession had structure and functionality comparable to that of the wild type. This observation is of significance to breeders who are working toward developing pennycress accessions with improved nutritional quality. Further, this research provided the first in vitro and in vivo comparisons of pennycress protein nutritional quality, revealing that defatted pennycress meal and salt extracted PcPI have PDCAAS comparable to or better than other plant proteins. While the functional properties of the PcPI produced in this study are acceptable, there is room for improvement via downstream protein modifications, or targeting breeding for high quality and content of cruciferin. Nevertheless, this research provided, for the first time, a comprehensive evaluation of optimized protein extraction methods to produce a functional PcPI that can compete with soy protein for various food applications, such as acidic beverages, meat and dairy products, and emulsified systems.

AUTHOR CONTRIBUTIONS

Rachel Mitaceka: Methodology, execution of experiments, investigation, formal data analysis, writing—original draft. **M. David Marksb:** Development of pennycress accessions. **Nicole Kerra, Daniel Gallahera:** In vivo and in vitro PDCAAS measurement. **Baraem P. Ismail:** Conceptualization, project management,

writing—review and editing, funding acquisition. All authors contributed to and approved the final draft of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

No human or animal subjects were used in this research.

ORCID

Baraem P. Ismail https://orcid.org/0000-0002-3551-8446

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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