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Protein Extraction and Membrane Recovery in Enzyme-Assisted Aqueous Extraction Processing of Soybeans

J. M. L. N. de Moura · K. Campbell · N. M. de Almeida · C. E. Glatz · L. A. Johnson

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Abstract Enzyme-assisted aqueous extraction processing (EAEP) is an environmentally friendly process in which oil and protein can be simultaneously recovered from soybeans by using water and enzymes. The significant amount of protein-rich effluent (skim) constitutes a challenge to protein recovery. Countercurrent two-stage EAEP at a 1:6 solids-to-liquid ratio, 50 °C, pH 9.0, and 120 rpm for 1 h was used to extract oil and protein from dehulled, flaked and extruded soybeans. Different enzyme use strategies were used to produce different skim fractions: 0.5% protease (wt/wt extruded flakes) in both extraction stages; 0.5% protease only in the 2nd extraction stage; and no enzyme in either stage. Dead-end, stirred-cell membrane filtration was evaluated with each skim. About 96, 89, and 66% of the protein were extracted with the three enzyme treatments, respectively. Protein retentate yields of 91, 96, and 99% were obtained for the three enzyme treatments, respectively, by using double membrane filtration (30 kDa/500 Da) of the skims, achieving permeate fluxes up to 1.24 kg/m^2 h at 3.9–4.8 concentration factors (CF) and 0.56 kg/m^2 h at 1.9–2.9 CF for 30 kDa ultrafiltration and 500 Da nanofiltration, respectively. For cross-flow ultrafiltration with the 3-kDa membrane, pH and presence of insoluble protein aggregates significantly affected permeate flux. Maximum permeate flux occurred at high pH and in the presence of protein aggregates, achieving a mean value of 4.1 kg/m^2 h at 1.7 bar transmembrane pressure.

Keywords Aqueous extraction · Soy protein · Membrane filtration · Soybeans

J. M. L. N. de Moura · C. E. Glatz · L. A. Johnson (✉)
Center for Crops Utilization Research, Iowa State University, Ames, IA 50011-1061, USA
e-mail: ljohnson@iastate.edu

K. Campbell · C. E. Glatz
Department of Chemical and Biological Engineering, Iowa State University, Ames, IA 50011-2230, USA

N. M. de Almeida
Departamente de Gestão e Tecnologia Agroindustrial, Universidade Federal da Paraiba, Bananeiras, PB 58220-000, Brazil

L. A. Johnson
Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011-1061, USA

Introduction

Soybeans are a major food and feed crop with about 240 million metric tons annual worldwide production. Currently the United States, Brazil, and Argentina account for 36, 25 and 21% of worldwide soybean production, respectively [1]. Soybean production is expected to increase due to growing demand for vegetable oils needed by the edible oil and biofuel sectors and for high quality protein needed by the livestock feeding industry [2]. Only about 2% of the available soy protein is consumed directly by humans in the form of soy products such as tofu, soy burgers, or soymilk and other dairy analogs. The remaining 98% is processed into soybean meal and fed to livestock, largely poultry and swine [3].

Enzyme-assisted aqueous extraction processing (EAEP) is considered to be an environmentally friendly process in which oil and protein can be simultaneously extracted from many oil-bearing materials by using water as an extraction medium [4–11]. The growing interest in vegetable proteins with good nutritional qualities and desirable functional properties as well as increasing safety concerns regarding

use of hexane in conventional oil extraction are driving extensive research into aqueous extraction processing of soybeans [12–17]. In addition to replacing the use of hazardous and polluting hexane, the use of this clean water- and enzyme-based technology enables simultaneous fractionation of soybeans into oil, protein and fiber-rich fractions suitable for converting into food, feed and fuel.

Some of the previous challenges of the EAEP of soybeans, such as low oil extraction yields and difficulty in de-emulsifying the cream fraction to obtain free oil, were solved by adopting mechanical treatments (flaking and extruding) and selecting better enzymes for extraction and cream de-emulsification steps [13, 14]. Oil and protein extraction yields of 96 and 85%, respectively, were obtained by using 0.5% protease and 1:10 solids-to-liquid ratio in single-stage extraction [13]. Despite high extraction yields obtained by single-stage EAEP of soybeans, the significant amount of oil that ends up in the large volume of the skim fraction (protein- and sugar-rich fraction) limits oil recovery (oil that can be recovered as free oil) to 82%. No economically viable method to recover the oil also present in the skim fraction (14%) has been developed. Recovering the protein from the large volume of skim is a significant challenge that impedes commercial adoption [17].

Countercurrent two-stage EAEP [16] reduced the amount of water used in single-stage EAEP by 40–50% and also improved oil and protein extraction yields. In addition, two different enzyme strategies were developed enabling the recovery of proteins with different degrees of hydrolysis and functionalities. Oil and protein extraction yields were 98 and 92%, respectively, when using a protease in both extraction stages, and 95 and 89%, respectively, when the enzyme was inactivated after the second stage but prior to the first stage of countercurrent extraction.

Scaling-up countercurrent two-stage EAEP from laboratory-scale experiments (0.08 kg of extruded flakes) to medium-scale experiments (2 kg of extruded flakes) produced similar oil (99%) and protein extraction (94%) values with slight modification in the oil distribution among the fractions [17]. More oil (23 vs. 13% of the total oil) was observed in the skim fractions at larger scale. Modifying extraction parameters from pH 8.0/15 min to pH 9.0/1 h improved the oil distribution among the fractions, increasing oil yield in the cream fraction from 76 to 86% and reducing the oil yield in the skim fraction from 23 to 12%. Oil and protein extraction yields of 99 and 96%, respectively, were achieved when using pH 9.0/1 h in both extraction stages.

We recently evaluated the effects of soybean moisture content (7.2–12.8%) and conditioning temperature (51–79 °C) for flaking on total oil and protein extraction yields, oil distribution among the fractions, and resistance of the cream to chemical and/or enzymatic de-emulsification [18]. Although protein extraction yield (~95%) was not significantly affected by soybean moisture and conditioning temperature, highest oil extractions (96.7–98.2%) were achieved when using soybeans containing from 8 to 12% moisture and were not affected by conditioning temperature. The best treatment combination is one giving high oil and protein extraction yields, high free oil yield, and low oil yield in the cream and skim fractions. The cream with high oil yield was easily demulsified compared with cream having a low oil yield (95 vs. 76.5% de-emulsification). Oil extractability (oil extracted from solids) achieved by countercurrent two-stage EAEP (95–99%) [16, 17], is as complete as commercial hexane extraction (95–98%) [19]; however, free oil recovery is reduced to (78–80%) because some oil is lost to the skim fraction. Using soybeans with 12% moisture content and conditioning at 75 °C was best for oil extraction, oil distribution among the fractions, and efficient enzyme use. Maximizing oil and protein recoveries are essential to improving economical viability of EAEP with soybeans.

Protein recovery from single-stage AEP and EAEP of extruded soy flakes using isoelectric precipitation [20, 21], ultrafiltration (UF), and ion-exchange chromatography [20] has been previously evaluated. Campbell and Glatz [20] using UF achieved optimal protein purity from skim having an intermediate extent of hydrolysis by using a 3-kDa membrane, with 0.74 protein retention coefficient and 70% retentate protein purity. Ion-exchange chromatography allowed separating protein from emulsified oil, recovering proteins with molecular weights between 12 and 30 kDa, but in low yields (15–20%). The best one-step protein recovery method from EAEP of extruded flakes was UF achieving 60–63% overall yields, although lower in protein purity than isoelectric protein precipitates.

The objectives of the present study were to: (1) evaluate different enzyme strategies to extract protein from soybeans when using countercurrent two-stage EAEP; (2) evaluate two-stage dead-end membrane filtration (ultra- and nanofiltration) of skim fractions to recover the protein present in each skim fraction; (3) determine the overall optimal process combination for protein recovery, incorporating both upstream extraction and downstream recovery methods; and (4) maximize permeate flux for cross-flow UF. Although oil and protein extractions should be maximized in order to improve the economic viability of the EAEP process, the main focus of this paper is on protein extraction and recovery. Considering that the choice of the best extraction strategy to achieve high protein extraction and recovery must also take into account oil extractability, oil extraction yields were determined and presented along with protein extraction data for each extraction strategy used.

Materials and Methods

Full-Fat Soybean Flakes

Full-fat soybean flakes were prepared from variety 92M91–N201 soybeans (Pioneer, a DuPont Business, Johnston, IA, USA) harvested in 2007. The soybeans were cracked into 4–6 pieces by using a corrugated roller mill (model 10×12SGL, Ferrell-Ross, Oklahoma City, OK, USA) and the hulls were removed from the meats (cotyledons) by aspirating with a multi-aspirator (Kice, Wichita, KS, USA). The meats were conditioned at 60 °C by using a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH, USA) and flaked to approximately 0.25 mm thickness by using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA, USA).

Extrusion of Soybean Flakes

The moisture content of the flakes was increased to 15% by spraying water onto the flakes while mixing in a Gilson mixer (model 59016A, St. Joseph, MO, USA). The moistened full-fat soybean flakes were extruded by using a twin-screw extruder (ZSE 27 mm diameter twin-screw extruder; American Leistritz Extruders, Somerville, NJ, USA). High-shear geometry screws were used in co-rotational orientation at 90 rpm screw speed. The extruder barrel (1,080 mm length) was composed of ten heating blocks that were set for the temperature profile 30–70–100–100–100–100–100–100–100–100 °C. The extruder was manually fed to achieve an output rate of 10.5 kg/h of extruded flakes. Based in our previous results [17], the flakes were not collected in water. The collets were cooled to room temperature, placed in polyethylene bags, and stored in a cold room at 4 °C until extracted. The extruded flakes contained 20.7 ± 1.5% oil (as is), 35.7 ± 0.5% protein (as is), and 11.3 ± 1.0% moisture.

Enzyme Treatment

Protex 6L, having minimum activity of 580,000 DU/g and obtained from Genencor Division of Danisco (Rochester, NY, USA), was used. Protex 6L is a bacterial alkaline endoprotease derived from a strain of *Bacillus licheniformis* and has its highest activity at pH 7.0–10.0 and 30–70 °C. The 0.5% enzyme dosage for the extraction was based on the weight of extruded flakes and was selected based on our previous work [13].

Countercurrent Two-Stage EAEP

Countercurrent two-stage EAEP was performed over four days. The extruded full-fat flakes were subjected to two-stage extraction and the liquid fraction (skim + cream + free oil) obtained from the second extraction stage of one trial was recycled to the first extraction stage of the next trial (incoming fresh flakes) on the following day (Fig. 1) to simulate countercurrent operation. In actual practice countercurrent two-stage EAEP would be a continuous process; four days were required in laboratory simulation only due to materials handing issues and complexity. On the first day of extraction, the first extraction-stage EAEP was performed with 1 kg of extruded flakes using 1:6 solids-to-liquid ratio. The slurry pH was adjusted to 9.0 with 2 N NaOH solution before adding 0.5% Protex 6L (wt/extruded flakes) and stirred for 1 h at 120 rpm and 50 °C. The reaction was carried out in a 20-L jacketed glass reactor. The slurry obtained in the first extraction stage was centrifuged at 3,000×*g* to remove the insoluble fraction. After removing the insoluble fraction, the liquid phase (skim, cream and free oil) was placed in a 5-L jacketed reactor used as a large separatory funnel and allowed to settle overnight at 4 °C. After settling, the liquid phase was separated into three fractions (skim, cream and free oil). The insoluble fraction obtained in the first extraction stage (1st insolubles) was then subjected to a second extraction stage. Prior to the second extraction stage, the 1st insoluble fraction was dispersed in water to

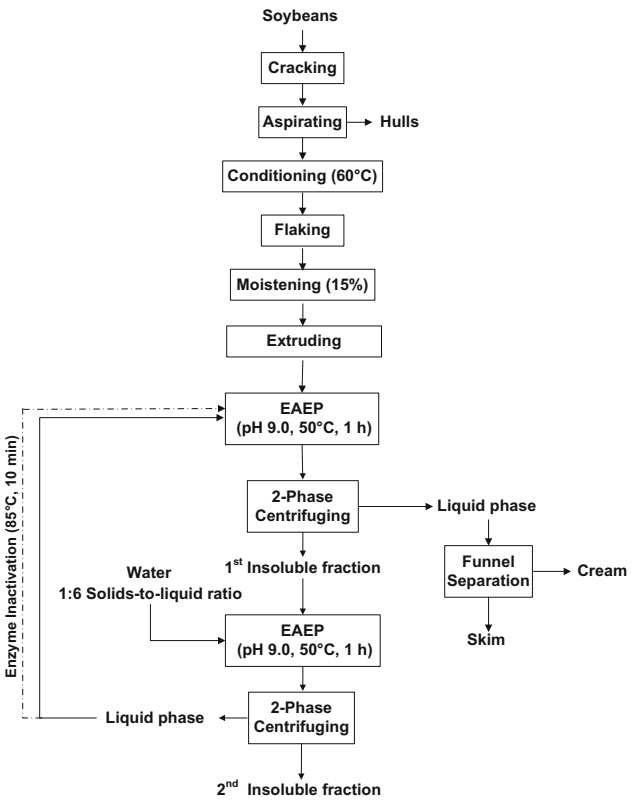

Fig. 1 Process flow diagram for countercurrent two-stage EAEP with two different enzyme strategies

obtain a 1:6 solids-to-liquid ratio and the same extraction conditions were used as in the first extraction stage. The slurry obtained in the second extraction stage was centrifuged to separate the insoluble and liquid fractions.

The liquid phase was recycled to the first extraction stage on the next day in two different ways: (1) without any heat treatment (active enzyme in both stages; Treatment 1); or (2) recycled liquid phase was heated for 10 min at 85 °C to inactivate the enzyme prior to the first-stage of countercurrent extraction (Treatment 2). Treatment 3 used the same recycle method as Treatment 1, but enzyme was not used in any extraction stage. Extractions were carried out in this manner for four consecutive days, with steady-state being achieved after the second day [17]. Samples of each fraction during the third and fourth extraction trials were collected and analyzed to determine chemical composition and mass balances of oil, protein, and solids were calculated based on incoming soybean flakes.

Oil, Protein and Solids Recoveries

Analyses of oil, protein and dry matter (solids) contents were carried out on the skim and insoluble fractions as well as the extruded full-fat flakes. Total oil contents were determined by using the acid hydrolysis Mojonnier method (AOCS method 922.06), protein contents by using the Dumas method and a nitrogen to protein conversion factor of 6.25 (vario MAXCN Elementar Analysensysteme GmbH, Hanau, Germany), and total solids by weight after drying the samples in a vacuum-oven at 110 °C for 3 h (AACC Method 44-40). Extraction yields were expressed as percentages of each component in each fraction relative to the initial amounts in the extruded full-fat flakes. Chemical analyses were performed in duplicate with samples obtained from two different extraction batches.

Size-Exclusion Chromatography of Skim Polypeptides

Low MW polypeptides were characterized by using high-performance liquid chromatography (HPLC) with a 300 mm × 7.8 mm Biobasic SEC 300 size-exclusion column (BioRad Laboratories, Ltd, Hercules, CA, USA). Samples were prepared in mobile phase buffer (2 M guanidine HCl) at about 1 mg/ml protein concentration and then filtered through a 0.45-μm regenerated cellulose membrane (Millipore Corporation, Billerica, MA, USA). Injection size was 10 μl at 1 mL/min mobile phase flow rate. Absorbance was measured at 215 nm. MW markers (Sigma, St. Louis, MO, USA) were aprotinin from bovine lung (6511 Da), insulin chain B (3,595 Da), angiotensin II human acetate (1,060 Da), and leucine enkephalin acetate hydrate (555 Da). The peptides were quantified based on their peak areas in relation to the standard markers.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

High MW profiles were determined by SDS-PAGE after diluting samples to 1.5 mg/ml protein concentration with 10 mM phosphate buffer (pH 7.5), mixing 1 part sample with 2 parts buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% 2-mercaptoethanol), heating in boiling water for 5 min, and loading onto a 4−15% gradient polyacrylamide gel (BioRad Laboratories, Ltd. Hercules, CA, USA) using 15-μl aliquots for loading 7.5 μg of protein.

Dead-End Ultra- and Nanofiltration of Skim Fractions

Laboratory-scale dead-end UF was performed by using Amicon stirred cells with 50- and 200-mL capacities (models 8,050 and 8,020; Billerica, MA, USA) and effective membrane areas of 13.4 and 41.8 cm^2, respectively. In the case of two-stage membrane filtration, 200 g of each skim fraction was ultrafiltered with 30-kDa regenerated cellulose (YM30) up to a concentration factor (CF) of 4.8 followed by nanofiltering a 50-g sample of the permeate with a 500-Da cellulose acetate (YC05) up to a CF of 2.8. Concentration factors (volume of feed/volume of retentate) ranged from 3.89 to 4.81 and 1.98 to 2.96 for the first and second stages, respectively. All membranes were manufactured by Millipore (Billerica, MA, USA) and each experiment was conducted in duplicate.

Analysis of Dead-End Permeates and Retentates

Permeates and retentates obtained after the first ultrafiltration (30 kDa) were analyzed for protein and solids contents. Additional filtration experiments were performed to obtain sufficient permeate to determine oil contents of both fractions and to obtain sufficient starting material for a second filtration with a 500-Da NF membrane. Yields of oil, protein, and solids were expressed as percentages of each component in each fraction relative to the initial amounts in the skim fraction. When nanofiltering with a 500-Da membrane, permeates were analyzed for protein content.

Protein Retention

The protein retention coefficient (R) was calculated according to Eq.1 [22]:

$$\log C_R/C_0 = R \log \mathrm{CF} \quad (1)$$

where C_R is the is the protein concentration in the retentate, C_0 is the initial feed concentration of protein, and R is the average retention of protein. Permeate samples were

collected at intervals during ultra- and nanofiltration for protein determination. Retentate protein content was determined by mass balance.

Membrane Fouling and Resistance

Global resistance was determined according to the Darcy equation:

$$J = \frac{\Delta P}{\mu_o \Sigma R} \quad (2)$$

where J is permeate flux, ΔP is transmembrane pressure, μ_o is permeate viscosity, and ΣR the global resistance is the sum of all resistances (i.e. membrane, fouling, cake formation, polarization, etc.). Rheological measurements were performed using a Haake RS150 Rheometer (Thermo-Orion, Karlsruhe, Germany) equipped with a DG41 sensor system following procedure described by Lakshmanan at al [23]. Clean membrane resistance was determined by measuring distilled water flux before sample ultrafiltration. Sample filtration was performed at a negligible concentration factor ($CF_{max} = 1.1$, after sampling was completed), by recycling the permeate to the filtration cell at transmembrane pressure values ranging from 0.7 to 4.1 bar. Fouled membrane resistance was determined by measuring clean water flux after filtering to a concentration factor of four and two for the 30-kDa and 500-Da membranes, respectively. Due to the higher extent of hydrolysis and higher probability to foul the membrane, skim from Treatment 1 was used to determine the fouling contribution on permeate flux reduction. Permeation of particles with sizes of the same order of magnitude as the membrane pore size could cause internal pore fouling due to deposition or adsorption of solutes thus reducing permeate flux [22]. Therefore, as a result of the similarity between the size of peptides in Skim 1 (<30 kDa) and the pore size of the 30-kDa membrane, greater fouling could be expected for the 30-kDa membrane than for the 500-Da membrane.

Cross-flow Ultrafiltration

The influence of flux conditions when using skim from Treatment 1 (without any heat treatment; active enzyme in both stages) were determined by using a two-level full-factorial experimental design with a 3-kDa nominal MW cut-off (NMWCO), Xampler™, hollow-fiber membrane cartridge (GE Healthcare, Piscataway, NJ, USA) consisting of 13 fibers 30 cm in length with a nominal fiber diameter of 1.0 mm and total area of 110 cm^2 operated in full recycle mode at room temperature. Cross flow rate and transmembrane pressure (TMP) were controlled by using a peristaltic pump and a needle valve on the retentate. Flux was measured by recording the rate of change in permeate mass for 3.0 min on a balance. Parameters studied are shown in Table 1. An insoluble precipitate formed in the skim at the extraction pH upon storage and the presence of this precipitate was included in the design. Insoluble precipitates that formed during refrigerated storage and on adjusting the pH to soy protein pI (pH 4.5) were removed by centrifuging at 4,000×g for 90 min (pH 8.9) or 15 min (pH 4.5) at room temperature in a swinging-bucket rotor. pH was adjusted by adding 2 N HCl.

Statistical Analyses

The experiment was a completely randomized design. The data were analyzed by analysis of variance (ANOVA) by using mixed models from the SAS system (version 8.2, SAS Institute, Inc., Cary, NC, USA). Means were compared using F-protected contrasts and the level of significance was set at $P < 0.05$. Statistical analysis for the full-factorial design experiment was completed using JMP 7.0 statistical software package by SAS, Inc.

Results

Extraction Yields of Protein, Oil and Solids

Figure 2 shows the effects of different enzyme treatments on extraction yields of oil, protein and solids during countercurrent two-stage EAEP of soybeans. Extraction yields of oil, protein and solids declined as enzyme use during extraction was reduced (means within same fraction were statistically different at $P < 0.05$). Higher oil and protein extraction yields (99.0 and 96%, respectively) were obtained when using enzyme in both extraction stages (Treatment 1) and were modestly reduced, although means were statistically different at $P < 0.05$, when using enzyme only in the second extraction stage (Treatment 2; 94 and 89%, respectively). These results are in agreement with those obtained at laboratory bench scale when using 0.08 kg of extruded flakes [16]. Oil and protein extraction yields of 98 and 92% were obtained when using enzyme in both extraction stages and 95 and 89% when enzyme was

Table 1 Parameters tested for optimizing ultrafiltration flux of EAEP skim

Parameter	Level 1	Level 2
Cross flow rate (L/min)	0.27	0.90
Transmembrane pressure (psi)	10	25
pH	4.5	8.9 (as extracted)
Presence of precipitate	No solids	With solids (as extracted)

used only in the second extraction stage [16]. The absence of enzyme in both extraction stages (Treatment 3) gave quite low oil and protein extraction yields, 84 and 66%, respectively. Although high oil and protein extraction yields are desirable, small reduction in protein extraction yields might be justified to produce soy protein products with different degree of hydrolysis and functionalities.

Soybean protein isolates (SPI) are usually prepared from flash-desolventized, hexane-extracted soy flour by alkaline extraction (pH 8−9) at low solids-to-liquid ratio (1:10). Protein extraction yields of 72% were achieved by Deak and Johnson [24] at extraction temperatures ranging from 25 to 60 °C. The treatment where no enzyme was used during extraction (Treatment 3) shows the effect of extrusion temperature (100 °C) on protein denaturation, reducing protein solubility and consequently protein extraction yield (66%). Using enzyme during extraction (Treatments 1 and 2) restored protein solubility thus improving protein extraction yields up to 96%.

Characterization of Skim Fractions Obtained by Different Extraction Treatments

Oil, Protein and Solids Contents of Skim Fractions

Similar to our observations for extraction yields of oil, protein and solids, increasing enzyme use during extraction increased protein and solids concentrations in the skim fractions (Table 2). Regardless of the enzyme treatment used, oil contents of the skim fractions were not statistically different. Since the same solids-to-liquid ratio (1:6) was used for all treatments, higher concentrations of protein and solids were observed in skim fractions having higher extraction yields.

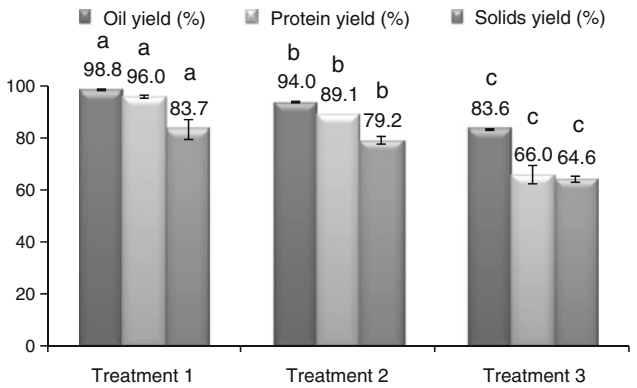

Fig. 2 Effects of enzyme treatment on extraction yields of oil, protein and solids in countercurrent two-stage EAEP. Treatment 1 [17], without any heat treatment (active enzyme in both stages; Treatment 1); Treatment 2, heated for 10 min at 85 °C to inactivate the enzyme prior to the first stage of countercurrent extraction; and Treatment 3, without enzymes in either extraction stages. Means within the same fraction with *different letters* above are statistically different at $P < 0.05$

The protein contents of skim fractions from Treatments 1 and 2 were 60.5 and 58% (dry-basis), respectively, with the most abundant impurities being emulsified oil and sugars. This is comparable purity to soy protein concentrates (SPC), which are typically 65% protein with the most abundant SPC impurity is fiber [25]. Still, protein extraction yields from EAEP were much greater than from conventional SPC processes, at 89−96% achieved for EAEP versus 60−70% typically achieved in industry for both SPC and soy protein isolates (a 90% purity protein product) [25]. Conditions that favored oil extraction also favored protein extraction, which was in agreement with our previous findings [16]. Regardless of extraction treatment, a nearly constant oil/protein ratio of ca. 1:10 was observed in all skims.

Size-Exclusion Chromatography of Polypeptides and SDS-PAGE

SEC profiles (Fig. 3) indicated different extents of protein hydrolysis for the three extraction treatments. Proteins >30 kDa were not resolved and appear as a large peak at short elution time. Skim of Treatment 3 has no hydrolyzed proteins; thus, no peptides between 11 and 1.5 kDa MW were observed. Very little unhydrolyzed protein remains in the skim of Treatment 1, but some proteins with MW >11 kDa likely remained unhydrolyzed. Although proteins with MW <20 kDa contain newly hydrolyzed peptides, it's possible that some proteins observed in the SDS-PAGE gel profiles (Fig. 4) are resistant to hydrolysis. The use of enzyme in both extractions (Treatment 1) reduced most peptides to MWs <20 kDa, while using enzyme only in the second extraction (Treatment 2) left more intact protein subunits compared with Treatment 3 (without enzyme use). The largest polypeptide in the skim of Treatment 1 is likely the basic subunit of glycinin, in agreement with findings reporting higher proteolysis resistance of the basic subunit of glycinin [27].

Disruption of protein networks, either by protein solubilization or protein hydrolysis, generally favors extraction of oil and protein [28–30]. More extensive protein

Table 2 Oil, protein and solids contents of skim fractions obtained from different enzyme use during countercurrent two-stage EAEP

Skim	Oil (%)	Protein (%)	Solids (%)
Treatment 1	0.62[a]	6.02[a]	9.95[a]
Treatment 2	0.49[a]	5.50[b]	9.47[b]
Treatment 3	0.46[a]	4.38[c]	8.41[c]

Means within the same column followed by different superscripts are statistically different at $P < 0.05$

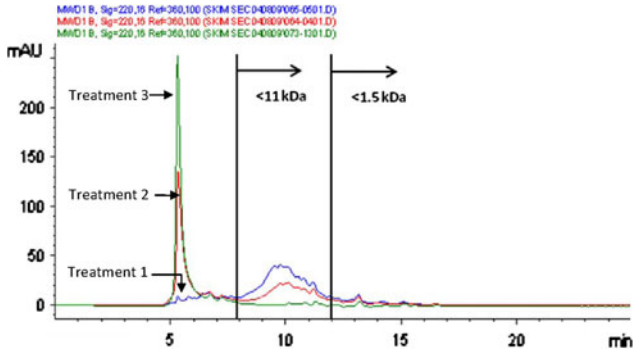

Fig. 3 SEC profiles of proteins in skims of Treatments 1, 2 and 3. Treatment 1, without any heat treatment (active enzyme in both stages; Treatment 1); Treatment 2, heated for 10 min at 85 °C to inactivate the enzyme prior to the first stage of countercurrent extraction; and Treatment 3, without enzymes in either extraction stages

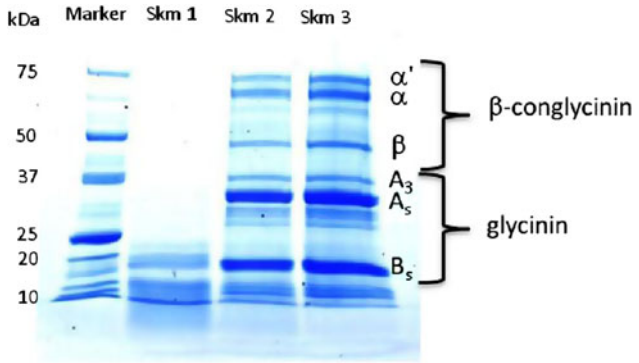

Fig. 4 SDS-PAGE gel of proteins in skims from the three extraction treatments. Major subunits of soybean storage proteins glycinin and *β*-conglycinin are indicated [26]

hydrolysis achieves higher extraction yields of oil and protein [13]. As can be seen in Figs. 2 and 4, higher oil and protein yields were also obtained from the treatments that achieved greater hydrolysis (Treatment 1 vs. Treatment 3).

Ultra- and Nanofiltration of EAEP Skim

Two-Stage Dead-End Membrane Filtration

Figure 5(a–c) shows the ultrafiltration of skims from Treatments 1, 2 and 3, respectively, when using a 30-kDa membrane at 4.14 bar. Permeate flux of skim of Treatment 1 decreased from 10.8 ± 1.0 to 1.2 ± 0.2 kg/m^2 h when the solids concentration in the retentate increased from 10.8 ± 0.2 to 23.4 ± 2.8%. At CF = 4.5, the highest CF that could be reached because the high viscosity of the retentate prevented stirring, protein yield in the retentate was 55.5 ± 1.6%. For skim of Treatment 2, permeate flux decreased from 5.1 ±0.1 to 0.5 ± 0.1 kg/m^2 h when solids concentration in the retentate increased from 9.7 ± 0.1 to 26.0 ± 1.2%. Protein yield in the retentate of 71.9 ± 1.5% was achieved at the highest CF (4.81). Permeate flux of skim from Treatment 3 decreased from 4.8 ± 0.3 to 0.4 ± 0.1 kg/m^2 h when solids concentration in the retentate increased from 8.7 ± 0.1 to 22.9 ± 0.3%. Protein yield in the retentate was 95.8 ± 0.7% at the highest CF (3.9). Permeate flux increased with increasing degree of protein hydrolysis (Treatment 1 > Treatment 2 > Treatment 3). Permeate fluxes were higher for skims with greater extents of hydrolysis, even though these skims had higher protein concentrations. Others have determined cake resistance to be the dominant source of resistance in ultrafiltration of SPCs [31]. Our extracts contained insoluble material in the range of 15 mg/mL, so cake layer formation was a possibility. Considering the high protein concentration, however, resistance due to concentration polarization was also likely to be high. Average protein retention for skims from Treatments 1, 2 and 3 were 0.62, 0.80 and 0.97, respectively (Fig. 6). Protein retentions were statistically different at $P < 0.05$. This is comparable to retentions of EAEP skim proteins achieved by Campbell and Glatz [20] who observed total protein retentions of 0.74 with a 3-kDa membrane. Reduced protein retention likely results in decreased concentration polarization at the membrane surface, allowing greater permeate flux. Trends in protein retention, as shown by the slopes of equations in Fig. 6, were consistent with the extents of hydrolysis of the skims, where increasing hydrolysis resulted in lower solute MW and lower protein retention. The effects of TMP on permeate flux of two-stage filtration (UF and NF) of skim from Treatment 1 (30-kDa and 500-Da membranes) are shown in Fig. 7. First stage filtration (30-kDa membrane) indicated that the critical TMP was below 0.7 bar (the lowest pressure tested), with flux only increasing marginally from 6.5 to 8 kg/m^2 h between 0.7 and 4.1 bar at CF < 1.1.

The 30-kDa membrane exhibited signs of compaction, with resistance increasing from 1.6×10^{12} and 2.7×10^{12} m^{-1} when increasing the TMP from 0.7 to 4.1 bar. After concentrating Skim 1 to CF = 4, fouled membrane resistances for two separate trials were 2.4×10^{12} and 3.3×10^{12} at 0.7 bar, and 4.0×10^{12} and 8.8×10^{12} m^{-1} at 4.1 bar, roughly doubling the membrane resistance compared to before ultrafiltration. Still, global resistances ranged from 4×10^{13} to 20×10^{13} m^{-1} at 0.7 and 4.1 bar, respectively. The fouled membrane resistances are therefore small (<7%) compared to the global resistances.

Proximate analysis of the retentates and permeates from ultrafiltration of skims from Treatments 1, 2 and 3 (Fig. 8) indicated that 92.6, 89.2 and 93.3% of the initial oil and 55.8, 73.4 and 95.8% of the initial protein present in respective skim fractions, respectively, remain in the retentate fraction. Regardless the extraction process, oil

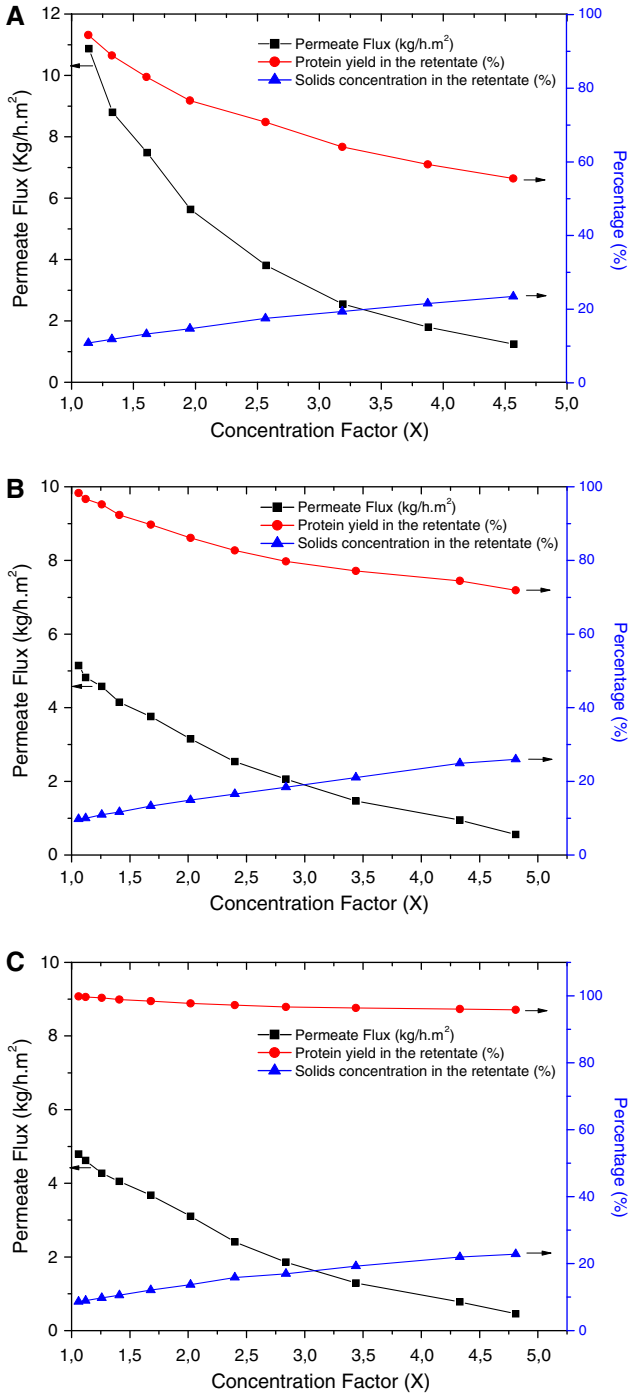

Fig. 5 Effects of concentration factor on permeate flux, protein yield and solids content in the ultrafiltration (30-kDa membrane, 4.14 bar) of skims from Treatments 1 (**a**), 2 (**b**) and 3 (**c**)

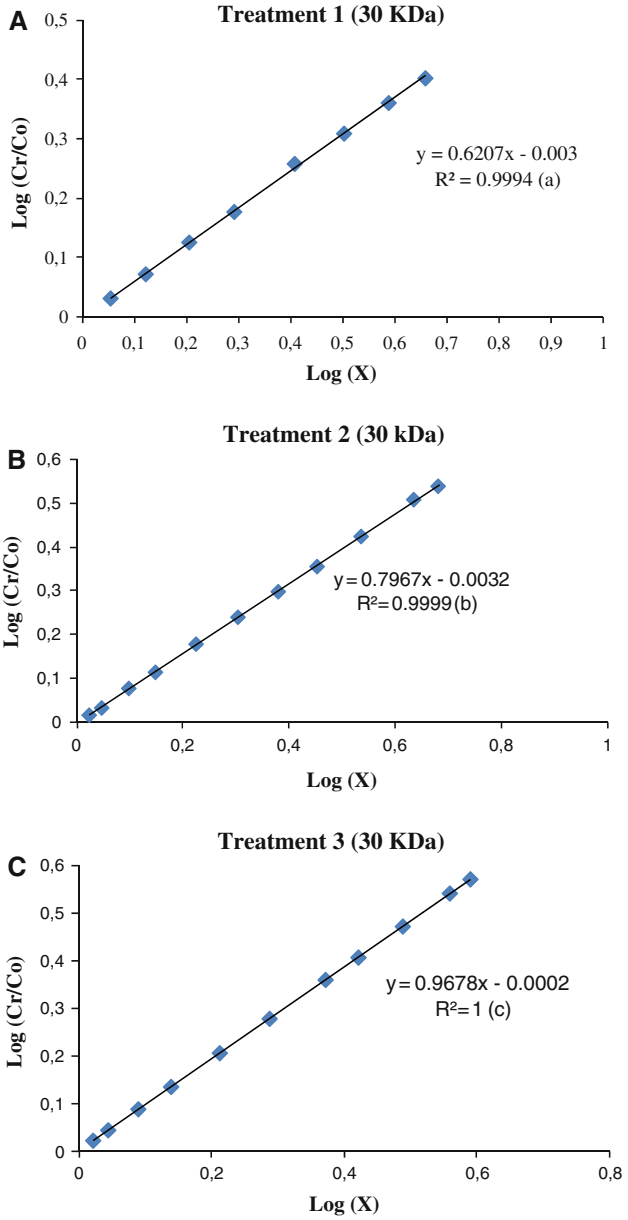

Fig. 6 Protein rejection during ultrafiltration (30-kDa membrane, 4.14 bar) of skims from Treatments 1, 2 and 3. Mean slopes followed by *different letters* are statistically different at $P < 0.05$

retention was not statistically different at $P < 0.05$, with between 7 and 11% of the oil passing through the membrane. The increased protein retention in the retentate, inversely related with degree of hydrolysis, was statistically different at $P < 0.05$ within all treatments. Although solids retention was not statistically different for Treatments 1 and 2, a significant increase in solids retention was observed for Treatment 3.

SEC profiles of retentates of skims from Treatments 1 (a), 2 (b) and 3 (c) (Fig. 9) indicate depletion of proteins <11 kDa for skims from Treatments 2 and 3. The SEC profile of skim of Treatment 3, which has very little protein <11 kDa and very high retention, did not substantially change during ultrafiltration. The retentate from skim of Treatment 1 has a considerable amount of proteins <11 kDa, but the relative quantity of the smaller polypeptides is much reduced compared to proteins >11 kDa.

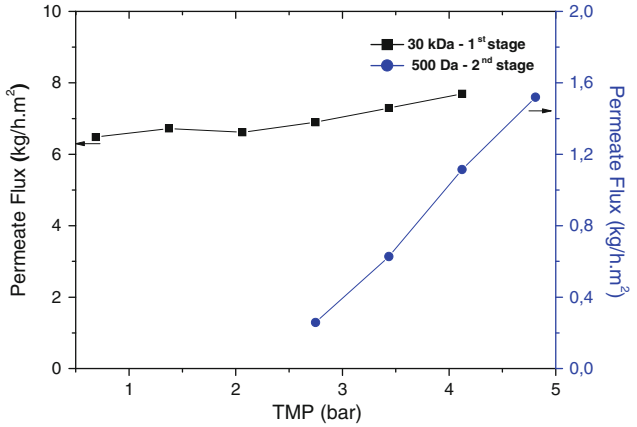

Fig. 7 Effects of transmembrane pressure on permeate flux of skim from Treatment 1 (first and second filtration stages, 30-kDa and 500-Da membranes, respectively)

Second-stage filtrations of permeates from ultrafiltration of skims of Treatments 1, 2 and 3 were performed with a 500-Da membrane at 4.8 bar (Fig. 10). Permeate flux of the first permeate from skim of Treatment 1 decreased from 1.2 ± 0.1 to 0.3 ± 0.1 kg/m^2 h at CF = 1.96. In the case of permeates from skims from Treatments 2 and 3, secondary permeate fluxes decreased from 3.6 ± 0.1 to 0.5 ± 0.1 kg/m^2 h and 5.6 ± 0.4 to 0.6 ± 0.1 kg/m^2 h at CFs of 2.13 and 2.96, respectively. At the final CFs, protein yields in retentate were 80.3 ± 2.6, 86.6 ± 1.8, and 83.3 ± 1.8% for skims of Treatments 1, 2 and 3, respectively.

An opposing trend was observed for permeate flux during second-stage filtration of permeates of skims from Treatments 1, 2, and 3 compared to first-stage filtration where flux increased with decreasing hydrolysis. Lower permeate fluxes were observed in secondary filtrations for samples with higher degrees of hydrolysis, which was likely a consequence of the higher protein contents in those samples. To illustrate these differences, the graphs in Fig. 10 were plotted in relation to protein yield in the retentate. Protein contents of permeates from ultrafiltered skims of Treatments 1, 2 and 3 (30-kDa membrane, first-stage filtration) were 3.6, 1.8 and 0.25%, respectively. Protein retention, as determined by Eq. 1, increased with increasing average MW, with retention values of 66.4, 79.1 and 83.7% for skims of Treatments 1, 2 and 3, respectively (Fig. 11). Statistical difference at $P < 0.05$ was observed when comparing protein retention of skim from Treatment 1 with protein retentions of skims from Treatments 2 and 3. Protein retentions of skims from Treatments 2 and 3 were not statically different at $P < 0.05$.

Measurements of flux as a function of TMP for the 500-Da ultrafiltration of the 30-kDa permeate of Skim 1 at CF < 1.1 indicated that 4.8 bar was below critical TMP

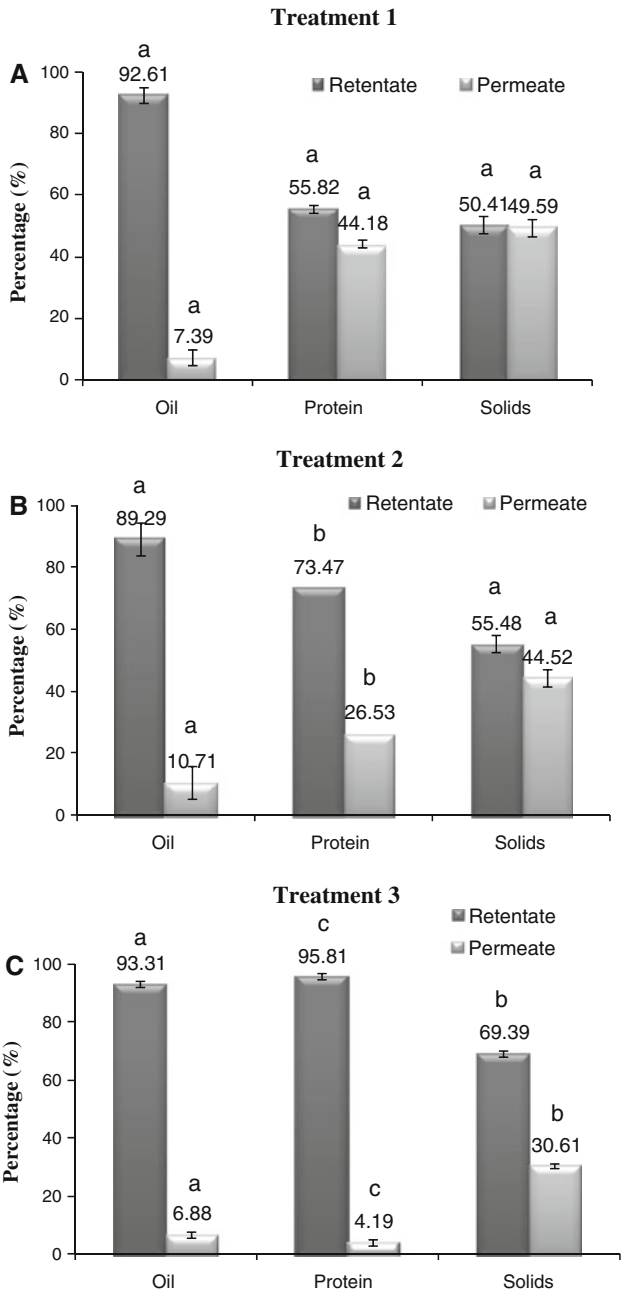

Fig. 8 Oil, protein and solids extraction yields in retentates and permeates from ultrafiltration (30 kDa) of skims from Treatments 1, 2 and 3. Means within the same fraction followed by *different letters* are statistically different at $P < 0.05$

(Fig. 7), although osmotic pressure of the bulk retentate solution for this case was high- extrapolation of the flux versus TMP curve crossed the *x*-axis at 2.4 bar suggesting an osmotic pressure of 2.4 bar. This would explain the severe flux decline seen by a CF of 2. It should be noted that the pressures used in the present study, which were limited by the experimental apparatus, were somewhat lower than what is commonly encountered in nanofiltration. Doubling

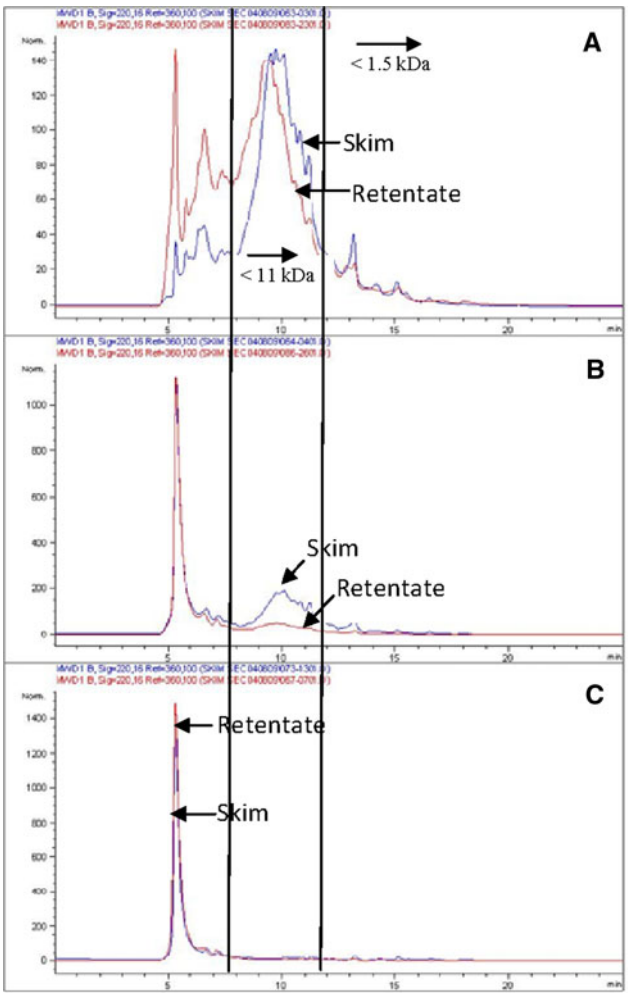

Fig. 9 SEC profiles of the skims and their retentates for Treatments 1 (**a**), 2 (**b**) and 3 (**c**) using a 30-kDa membrane. Chromatograms are normalized for the largest peak, and the relative peak sizes between chromatograms are not directly comparable

the retentate concentration (and, hence at least doubling the osmotic pressure) would reduce the effective TMP (ΔP, osmotic pressure) to near zero. After concentrating the sample to a CF = 2, the fouled membrane resistance was on average 6.8×10^{13} m^{-1}, slightly greater than the clean membrane resistance, which was about 6.3×10^{13} m^{-1}. Fouled membrane resistance was about 10% of global resistance which, using an osmotic pressure of 2.4 bar, ranged from 6.2×10^{14} to 6.7×10^{14} m^{-1}.

Considering that 55.8% of the initial protein of skim of Treatment 1 was concentrated in the first filtration (CF = 4.5) and 80.3% of the initial protein of permeate from skim of Treatment 1 was concentrated in the second filtration (CF = 1.96), overall protein recovery in retentates reached 91.3% in relation to the initial amount of protein in the skim fraction. For skim of Treatment 2, protein yields in the retentates for both filtrations (73.5 and 86.5%, respectively) achieved 96.4% overall protein recovery. Protein

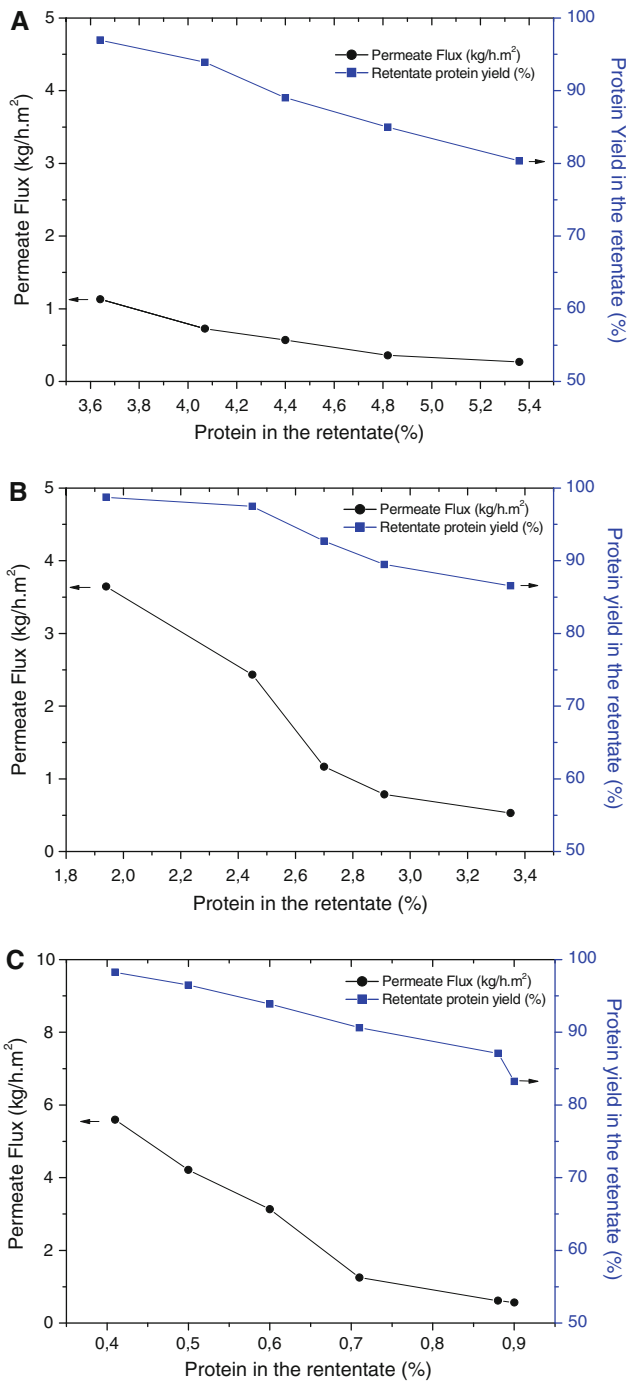

Fig. 10 Permeate fluxes and protein yields in the retentate from nanofiltering (500 Da, 4.81 bar) the permeates of skims from Treatments 1, 2 and 3

yields of 95.8 and 83.2% were obtained with successive filtrations of the skim of Treatment 3 achieving 99.3% overall protein recovery. Integrating different enzymatic treatments and successive membrane filtrations enabled 87.6, 85.9, and 65.5% of the initial protein in the extruded flakes to be recovered for Treatments 1, 2 and 3, respectively. Protein contents of concentrates from the first and

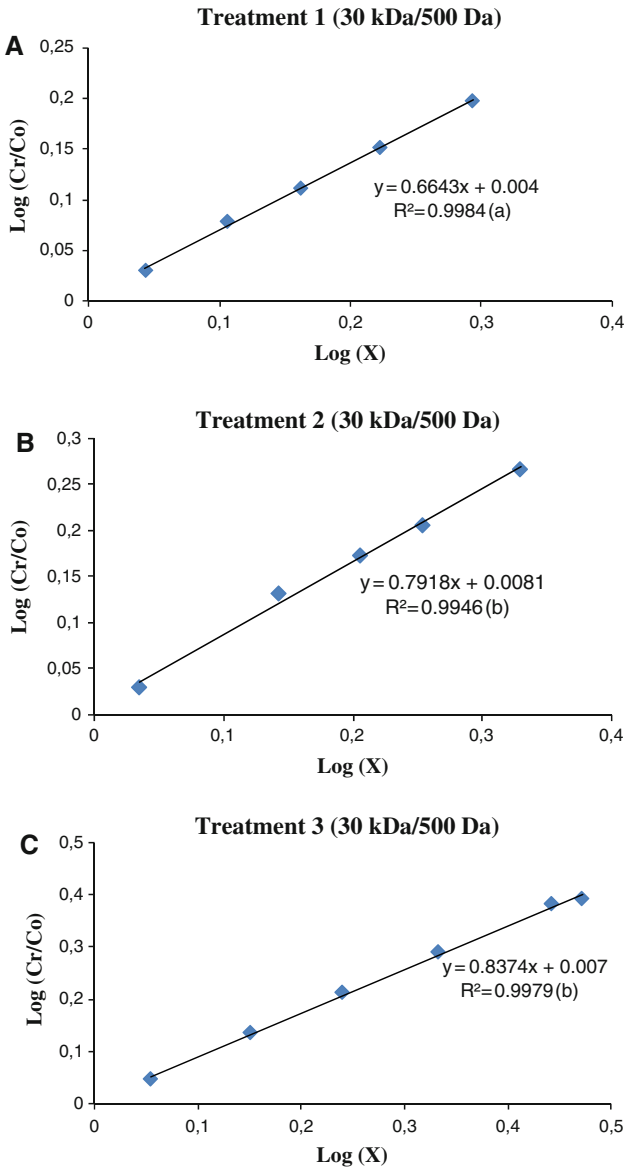

Fig. 11 Protein rejection during nanofiltration (500 Da, 4.81 bar) of the permeates from skims of Treatments 1 (**a**), 2 (**b**) and 3 (**c**). Mean slopes followed by *different letters* are statistically different at $P < 0.05$

second filtrations were 15.9, 19.9 and 17.8% and 5.4, 3.3 and 0.9% for skims of Treatments 1, 2 and 3, respectively. The concentrated protein retentates still need to be evaporated to remove the remaining water. Concentrated protein retentates from first and second filtrations contain 74.9, 72.7 and 76.8% and 86.8, 93.1 and 93.7% moisture for skims of Treatments 1, 2 and 3, respectively. However, the amount of water to be evaporated in both the first and second retentates at the respective CFs would be 56, 54 and 46% of the amount of water to be evaporated from the skims from Treatments 1, 2 and 3, respectively.

Table 3 Parameter estimates and significance test for effects of transmembrane pressure (TMP), cross flow rate (CFR), presence or absence of insolubles, and pH (4.5 or 8.9)

Parameter	Estimate	$P < t$
Intercept	2.5	<0.001*
TMP	1.18	<0.001*
CFR	0.17	<0.001*
Insolubles (absent)	−0.09	0.005*
pH (4.5)	−0.14	<0.001*
TMP × CFR	0.054	0.031*
TMP × insolubles (absent)	−0.016	0.5
TMP × pH (4.5)	−0.11	<0.001*
CFR × insolubles (absent)	0.040	0.11
CFR × pH (4.5)	0.015	0.55
insolubles × pH (4.5)	−0.056	0.02*

* significance at $P < 0.05$

Conditions Affecting Ultrafiltration Flux in a Cross-Flow System

The objective of the full-factorial design experiment was to determine how UF conditions within the range of parameters tested above affect flux. Even at high pH, insoluble protein aggregates formed upon storage, making up 12.8% of total solids in the skim for a concentration of 14 mg/mL and a soluble protein concentration of 58.9 mg/mL. At pH 4.5, precipitate accounted for 16.1% of total solids, for a concentration of 17 mg/mL, and a soluble protein concentration of 50.0 mg/mL. Therefore, pH, cross flow rate (CFR), TMP and the presence of insoluble aggregate material at existing pH and at pH 4.5 were investigated using skim from Treatment 1.

All four parameters significantly affected flux (Table 3). Maximum flux occurred at high CFR, high TMP, high pH, and in the presence of solids at room temperature. Significant interactions occurred between TMP and CFR, TMP and pH, and insolubles and pH, which can be seen in interaction plots (Fig. 12). Increasing CFR increased flux more at higher TMP. This was likely because at low TMP, polarization concentration would not be as extreme as at high TMP (hence, higher flux). Since the CFR affects flux by reducing the gel polarization layer thickness, it follows that the effect of the CFR would be reduced when concentration polarization was less. The increase in flux attributed to increased TMP was less at pH 4.5 than at pH 8.9. The presence of insolubles had little effect at high pH, but was very pronounced at pH 4.5 even though the insolubles concentrations for the two samples differed by only 3 mg/mL (14 vs. 17 mg/mL). At pH 8.9, the skim had a volume-weighted mean particle diameter of 23 μm, comparable to the mean particle size of the pH 4.5 skim,

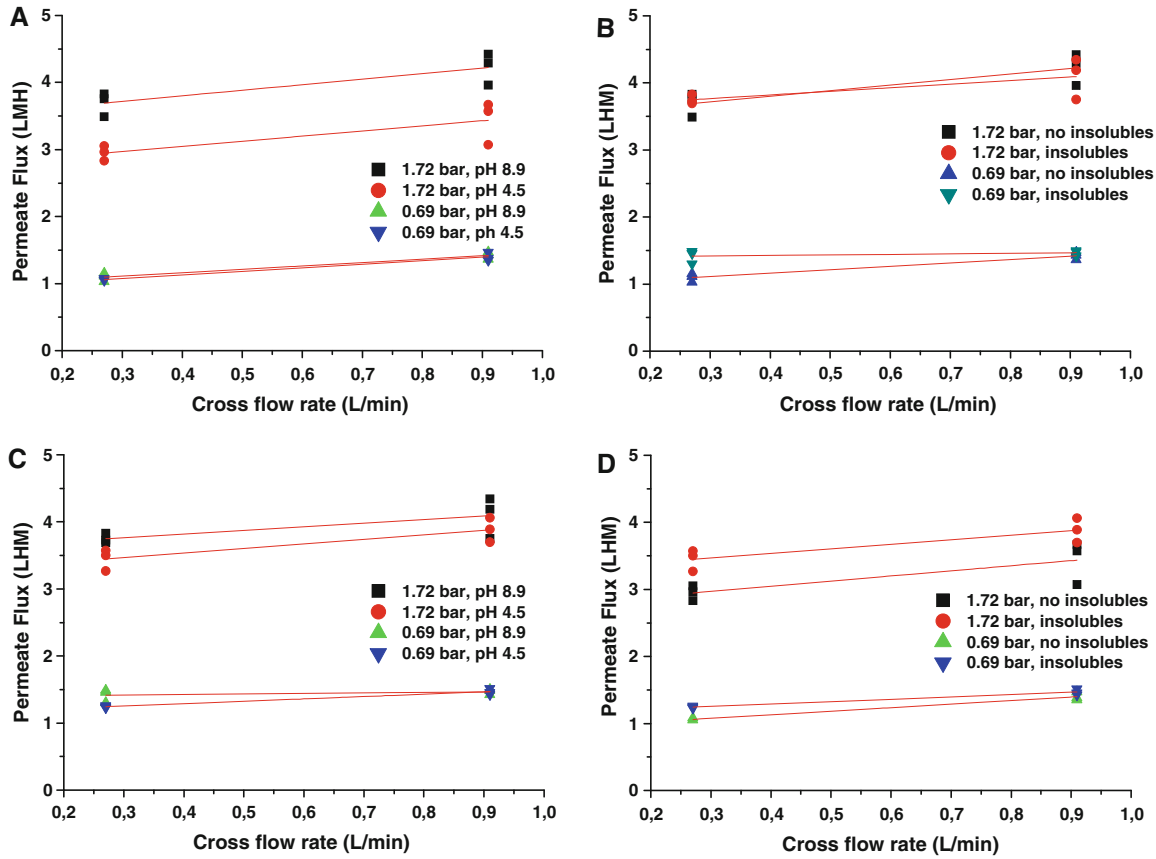

Fig. 12 Interaction plots of flux for ultrafiltration (3-kDa membrane) with: a no insolubles, pH 4.5 and 8.9; b pH 8.9 with and without insolubles; **c** with insolubles at pH 4.5 and 8.9; d pH 4.5 with and without insolubles

which was 33 and 17 μm before and after ultrafiltration, respectively. These are all in the particle size and concentration ranges where particles contribute to shear-induced diffusion near the membrane wall, leading to increased flux [32]. It is possible that emulsified oil remaining in suspension at high pH may contribute to shear-induced diffusion even after removal of other insolubles. Membrane resistances were between 1.8 and 2.2 $\times$ 10^{13} m^{-1} for all experiments. Fouling resistance increased with filtration time, ranging from 1.7 $\times$ 10^{13} m^{-1} for filtrations of less than 1 h to between 3.5 and 4 $\times$ 10^{13} m^{-1} for filtrations of 3−4 h. By comparison, global resistances for CFR of 0.27 L/min were between 18 and 20 $\times$ 10^{13} m^{-1}. Therefore, membrane and fouling resistances accounted for 20–33% of global resistance.

Conclusions

The increased use of enzyme in two-stage EAEP improved oil and protein extraction yields as well as protein hydrolysis. Oil, protein and solids contents were higher in skim fractions produced with increased enzyme use. Overall protein retentate yields when using double membrane filtration of skims was affected by protein hydrolysis. Greater hydrolysis was associated with less protein retention and higher permeates fluxes. Integrating different enzymatic treatments and successive membrane filtrations enabled 87.6, 85.9 and 65.5% of the initial protein in the extruded flakes to be recovered for Treatments 1, 2 and 3, respectively. For all dead-end filtrations, high protein retention was associated with low permeate flux. pH and the presence of insoluble protein aggregates significantly affected flux. Maximum flux occurred at high pH and in the presence of protein aggregates.

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