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Enzyme-Assisted Aqueous Extraction of Oil and Protein from Soybeans and Cream De-emulsification

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Abstract The effects of two commercial endoproteases (Protex 6L and Protex 7L, Genencor Division of Danisco, Rochester, NY, USA) on the oil and protein extraction yields from extruded soybean flakes during enzyme-assisted aqueous extraction processing (EAEP) were evaluated. Oil and protein were distributed in three fractions generated by the EAEP: cream + free oil, skim and insolubles. Protex 6L was more effective for extracting free oil, protein and total solids than Protex 7L. Oil and protein extraction yields of 96 and 85%, respectively, were obtained using 0.5% Protex 6L. Enzymatic and pH treatments were evaluated to de-emulsify the oil-rich cream. Cream de-emulsification generated three fractions: free oil, an intermediate residual cream layer and an oil-lean second skim. Total cream de-emulsification was obtained when using 2.5% Protex 6L and pH 4.5. The extrusion treatment was particularly important for reducing trypsin inhibitor activity (TIA) in the protein-rich skim fraction. TIA reductions of 69 and 45% were obtained for EAEP skim (the predominant protein fraction) from extruded flakes and ground flakes, respectively. Protex 6L gave higher degrees of protein hydrolysis (most of the polypeptides being between 1,000 and 10,000 Da) than Protex 7L. Raffinose was not detected in the skim, while stachyose was eliminated by α-galactosidase treatment.

Keywords Aqueous processing · Protein · Oil · Soybeans · Extraction · Enzymes

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Introduction

Currently, most soybean oil extraction is carried out by direct solvent extraction of uncooked soybean flakes. The use of a petroleum distillate containing about two-thirds *n*-hexane is typically used in the commercial extraction of soybean oil. Residual oil contents of solvent-extracted soybean meal are <1% [1]. There has been much concern regarding safety and environmental emissions associated with hexane usage. The Environmental Protection Agency has identified solvent emissions in oilseed extraction to be a significant source of air pollution, and has issued restrictive regulations on hexane emissions [2]. To reduce hexane emissions, alternative methods for edible oil extraction have been proposed [3, 4].

The aqueous extraction process (AEP), in which oil extraction is based on the insolubility of oil in water rather than on the dissolution of oil, is one such alternative [3, 4]. In AEP, oil and protein are extracted from the high-fiber solids and the extraction mixture is centrifuged to produce oil-rich (free oil and cream emulsion), oil- and protein-lean spent solids, and a protein- and sugar-rich aqueous phase (skim) [5]. AEP offers several advantages over conventional solvent extraction—less capital investment, inherently safe operation, and simultaneous production of edible oil and protein-rich fractions with less protein damage. The challenges when

using this process are to improve the efficiency of oil extraction, to effectively de-emulsify the difficult-to-break cream in order to recover free oil when emulsions are formed, and to develop high-value uses for the dilute protein-rich aqueous effluent (skim) [3, 5].

The mechanisms of oil and protein extraction into aqueous media from soybean flour have been determined by Rosenthal et al. [6]. Protein and oil extraction yields were shown to be closely related, with both depending on the extent of cell wall disruption. The conditions that favored protein extraction (alkaline pH, small particle size, and temperature below the level that causes denaturation) also favored oil extraction. Protein and oil extraction yields of ~65% have been obtained at pH 8.0, a solids-to-liquid ratio of 1:10, 50 °C, for 1 h of extraction and 200 rpm agitation.

Enzyme treatment has been used to increase oil extraction yield in AEP to as much as 90% [7–10]. Enzymes (cellulases, hemicellulases, and pectinases) are helpful for breaking down the structures of cotyledon cell walls and lipid body membranes (proteases). Proteolytic enzymes seem to be effective in hydrolyzing the oleosins, the lipophilic protein surrounding lipid bodies, thereby decreasing the surface activity of oleosin and enabling removal of lipid [3]. Various forms of the enzyme-assisted aqueous extraction process (EAEP) have been investigated for several oil-bearing materials such as soybeans [7], corn germ [8], rapeseed [10], coconut [11], rice bran [12], and sunflower [13].

The low oil recovery in AEP and EAEP has been related to the inadequacies of pretreatments at disrupting the cellular structure of oil-bearing materials [9]. Mechanical and heat treatments have been used to improve the rupture of cell walls, facilitating further enzyme degradation of the cell walls. Rosenthal et al. [6] reported that the oil extraction recovery increased from 22 to ~65% when the particle size of full-fat soy flour was reduced from 1,200 to ~100 μm. The effects of flaking and extruding have been evaluated as a means of enhancing oil extraction during EAEP [14]. Extruding soybean flakes increased the oil extraction from 46 to 71% in AEP. In EAEP, protease action was favored by extrusion, increasing oil recovery from 56 (unextruded flakes) to 88% (extruded flakes).

Lamsal and Johnson [15] reported that the total oil recovery from extruded soybean flakes in EAEP (88%) was distributed into three fractions: free oil, skim and cream, containing 16.0, 13.0, and 60% of the total oil, respectively. An important challenge to be overcome in the EAEP is the de-emulsification of the cream to obtain free oil. Enzymatic (phospholipases) and nonenzymatic (heating at 95 °C for 5 h, freeze–thawing) treatments have also been evaluated by Lamsal and Johnson [15]. The enzyme treatment achieved similar amounts of recoverable free oil to freeze–thawing, yielding 70–80% of total oil in the full-fat flakes. The ability to obtain high amounts of free oil resulted from a combination of extruding the flakes and the de-emulsification treatment. Heating for up to 5 h at 95 °C did not break the emulsion.

Because of the mild processing conditions of EAEP, the resulting skim fraction of soluble protein has the potential to be a valuable co-product [3, 7]. Soybeans contain anti-nutritional factors, such as trypsin inhibitor and flatus-producing oligosaccharides (stachyose and raffinose), which reduce the value of soy protein as food and feed ingredients [16]. Proteolytic treatments have been shown to reduce trypsin inhibitor activity (TIA) in soybean products [17], as well as to increase the digestibility of soy protein by early-weaned pigs [18]. Stachyose and raffinose levels of soy protein extracts have also been effectively reduced using ultrafiltration [19, 20]. Proteolytic treatments, however, may reduce the size of polypeptides such that they are close in size to the oligosaccharides, reducing the efficiency of ultrafiltration separation.

The objectives of the present study were: (1) to verify the effectiveness of two proteases, Protex 6L (P6L) and Protex 7L (P7L), in EAEP using extruded full-fat soybean flakes; (2) to evaluate enzymatic and chemical de-emulsification treatments of the cream using both proteases; (3) to characterize the extruded EAEP skim proteins, and; (4) to investigate the reduction of stachyose in the protein-rich skim by ultrafiltration and carbohydrase treatment.

Materials and Methods

Full-Fat Soybean Flakes

Full-fat soybean flakes were prepared from variety 92M91-N201 soybeans (Pioneer/DuPont, Johnston, IA, USA) harvested in 2006. The soybeans were cracked (model 10X12SGL, Ferrel-Ross, Oklahoma City, OK, USA) and aspirated (multi-aspirator, Kice, Wichita, KS, USA) to remove hulls, and the meats were conditioned at 60 °C (triple-deck seed conditioner, French Oil Mill Machinery Co., Piqua, OH, USA). The conditioned meats were flaked to approximately 0.25 mm of thickness using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA, USA). The initial moisture content of the flakes (9.6%) was increased to 12% by spraying water while mixing the beans in a Gilson mixer (model 59016A, St. Joseph, MO, USA). The conditioned flakes contained 21.0% oil (as is), 32.0% protein (as is) and 12.0% moisture.

Extrusion and EAEP Simulation

Soybean flakes were extruded at 100 °C barrel temperature and 100 rpm screw rotational speed with a high-shear

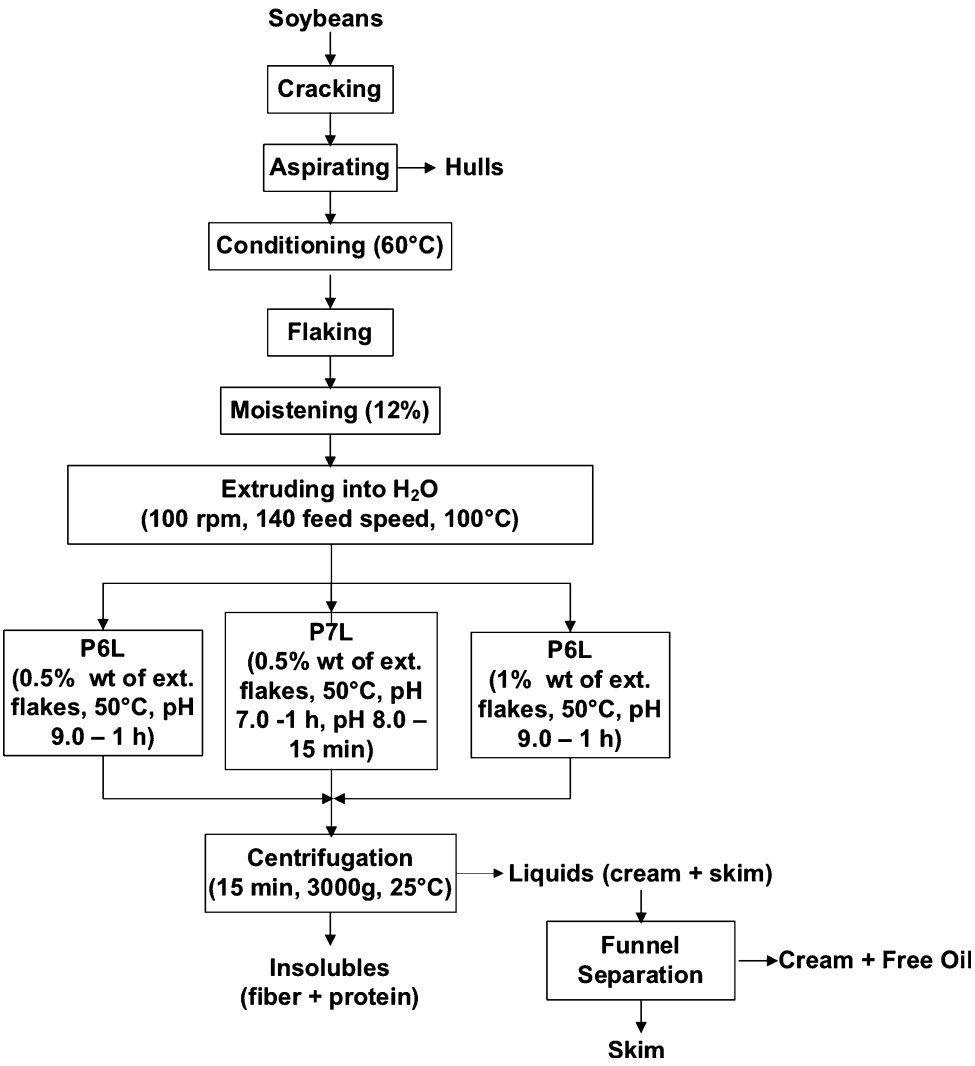

Fig. 1 Process flow diagram for enzyme-assisted aqueous extraction of extruded soybean flakes

geometry screw in a twin-screw extruder (18-mm screw diameter, Micro 18, American Leistritz Extruders, Somerville, NJ, USA). About 80 g of extruded flakes were collected directly into water in a 1-L beaker. Additional water was added to achieve a 1:10 solids-to-liquid ratio.

Two endoproteases obtained from Genencor International (Rochester, NY, USA) were evaluated in the EAEP: (1) Protex 7L, a bacterial neutral protease with endopeptidase activities derived from *Bacillus amyloliquefaciens*; and (2) Protex 6L, a bacterial alkaline protease derived from a selected strain of *Bacillus licheniformis*. The optimum pH and temperature ranges for the activities of both proteases were pH 6.0–8.0 and 40–60 °C (Protex 7L) and pH 7.0–10.0 and 30–70 °C (Protex 6L). Enzyme concentrations of 0.5% Protex 7L and 0.5 and 1.0% Protex 6L were evaluated. The enzyme dosage used in extraction was based on the weight of extruded flakes. For Protex 7L, the slurry pH was maintained at 7.0 while stirring for 1 h. Afterwards, the slurry pH was adjusted to 8.0 and stirred for an additional 15 min. For Protex 6L, the slurry pH was adjusted to 9.0 and stirred for 1 h. All extractions were carried out at 50 °C. Following extraction, the slurry was centrifuged at 3,000×*g*. A process flow diagram for EAEP of extruded soybean flakes is shown in Fig. 1.

Oil, Protein, and Solids Recoveries

Analyses of oil, protein, and dry matter contents were carried out on the skim, insoluble and cream fractions, as well as the initial extruded flakes. Total oil contents were determined by using the acid hydrolysis Mojonnier method (AOCS method 922.06), the protein contents by using Kjeldahl method (AACC Standard Method 46-08), and the total solids (dry matter) by weighing after drying the samples in a vacuum-oven at 110 °C for 3 h (AACC Method 44-40). The extraction yields were expressed as percentages of each component in each fraction relative to the initial amounts in the extruded flakes. Each enzyme treatment was replicated four times, with each replication being a different extrusion. Statistical analysis was

evaluated by the SAS system (version 8.2, SAS Institute, Inc., Cary, NC, USA) at $P < 0.05$. Three EAEP trials were carried out for each extrusion.

De-emulsification of Cream

The de-emulsification step was applied to the [cream + free oil] fraction obtained from EAEP using 0.5% Protex 6L (P6L cream) and 0.5% Protex 7L (P7L cream). For the enzyme treatment, 20 g of [cream + free oil] was adjusted to pH 9.0 with 2 N NaOH in a 30-mL beaker before adding 2.5% Protex 6L (w/w). The reaction was carried out at 50 °C with constant stirring using a ThermoScientific Variomag multipoint inductive-drive stirrer with external control (ThermoScientific, Daytona Beach, FL, USA) submerged in a water bath for 90 min. For the pH treatment, the same amount of sample was adjusted to pH 4.5 using 2 N HCl. Once the pH was adjusted, the samples were incubated at different temperatures and for different times.

At the end of the de-emulsification treatment, the samples were transferred to a 50-mL centrifuge tube and centrifuged at 3,000×*g* for 15 min at 20 °C. Three distinct layers were obtained (free oil, an intermediate layer, and a water phase referred to as second skim). The intermediate layer was located between the free oil and the second skim fraction. This intermediate layer is the remaining cream layer present after the de-emulsification treatment. Most of the free oil was collected by using a Pasteur pipette, and the remaining free oil was rinsed two times using hexane, following methods described by Lamsal and Johnson [15]. After evaporating the hexane, the weight of each fraction was recorded. Hexane was used only to accurately quantify the free oil; we do not envision using hexane in commercial practice. The free oil yield (%) was calculated as follows: free oil yield = [free oil (g) + hexane-washed free oil (g)]/[cream (g) × oil content (%) in {cream + free oil fraction}]. A process flow diagram for de-emulsifying the cream and a picture of the three fractions obtained after centrifugation are shown in Fig. 2.

Trypsin Inhibitor Activity in Skim from EAEP of Extruded Flakes

Prior to analysis, the skim fraction was filtered through a 0.45-μm membrane to improve clarity and diluted with 0.9% (w/v) NaCl solution to achieve 40–60% inhibition in the assay. TIA was characterized by using a modified assay for aprotinin from Sigma [21]. The assay is based on the spectrophotometric rate determination of the cleavage of a synthetic substrate, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), by trypsin with and without inhibitor present. BAPNA solution [1.00 mL of 0.1% (w/v)] was added to 1.60 mL of buffer (200 mM triethanolamine with 20 mM $CaCl_2$, pH 7.8) with 0.20 mL trypsin (0.16 mg/mL in 1.0 mM HCl) and 0.20 mL skim fraction in a cuvette and placed immediately in the spectrophotometer (Ultraspec 4000, Pharmacia Biotech, Piscataway, NJ, USA). The initial rate of change of absorbance at 405 nm was recorded. TIA was calculated as shown in Eq. 1:

$$\mathrm{TIA} = \frac{\left(\Delta A_{405\,\mathrm{nm}}/\Delta t_{\mathrm{uninhibited}} - \Delta A_{405\,\mathrm{nm}}/\Delta t_{\mathrm{sample}}\right)(\mathrm{df})}{9.96(0.2\,\mathrm{mL\ sample}/3\,\mathrm{mL\ reaction\ mix})} \quad (1)$$

Here, TIA is the trypsin inhibitor activity (unit/mL), $\Delta A_{405\mathrm{nm}}/\Delta t$ is the rate of change in absorbance per minute,

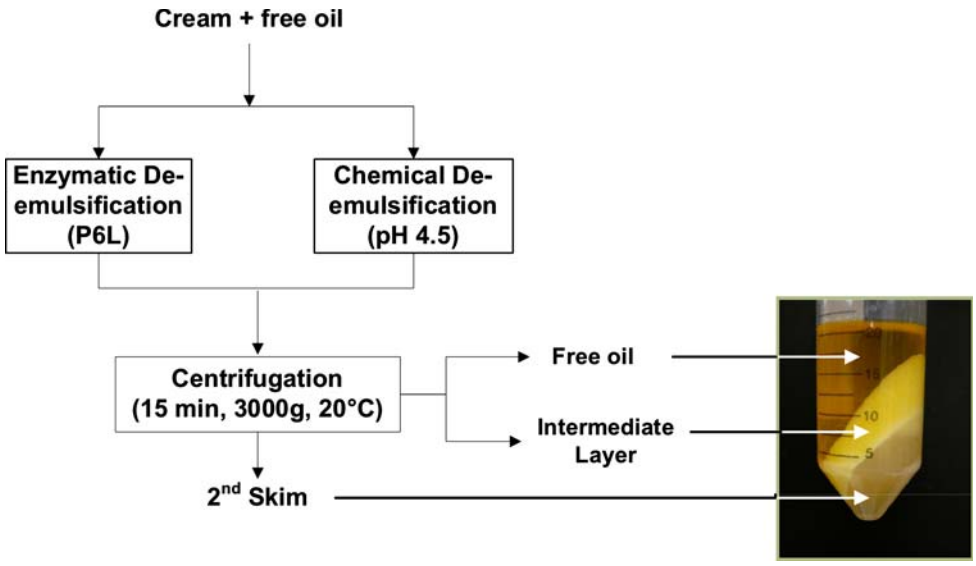

Fig. 2 Flow diagram for cream de-emulsification

df is the dilution factor, and 9.96 is the millimolar extinction coefficient for the BAPNA cleavage product. The uninhibited sample was 0.20 mL of NaCl solution.

Amino Acid Analysis

Amino acid analysis was conducted by the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) following AOAC Official Method 982.30 E(a,b,c), Chap. 45.3.05 (2006).

SDS PAGE

High-MW profiles were determined by SDS PAGE on a 4–15% gradient polyacrylamide gel (BioRad Laboratories, Ltd., Hercules, CA, USA). For each sample, 20 μL were diluted to 1 mL with de-ionized water. Each well was loaded with 10 μL of diluted sample for protein loadings of 6 and 8 μg for extruded skim and flaked flour skim samples, respectively. For comparison, soy protein isolate (ProFam® 646; Archer Daniels Midland Company, Decatur, IL, USA) was dissolved in 0.1 M phosphate buffer at pH 7.5 for a concentration of 1 μg/mL and loaded onto the gels with a 10-μL aliquot.

HPSEC of Polypeptides

Low-molecular-weight distributions were determined by high-performance size-exclusion chromatography (HPSEC) using a 300 mm × 7.8 mm Biobasic SEC 120 column (BioRad Laboratories, Ltd.). Molecular weight markers were: aprotinin from bovine lung (6,511 Da), insulin chain B (3,595 Da), angiotensin II human acetate (1,060 Da), and leucine enkephalin acetate hydrate (555 Da), all from Sigma (St. Louis, MO, USA). Fifteen microliters of each sample were diluted to 1 mL with water purified to a conductivity of 6.6 μS/m. Samples were then filtered through a 0.45-μm regenerated cellulose membrane (Millipore Corporation, Billerica, MA, USA). Mobile phase was 0.1 M phosphate buffer at pH 7.5. Injection size was 10 μL for loading about 5 μg of protein, with a mobile phase flow rate of 1 mL/min. Absorbance was measured at 215 nm.

HPLC of Raffinose and Stachyose

Raffinose and stachyose concentrations were determined by monitoring the refractive indices of samples eluted from a 300 mm × 7.8 mm Aminex HPX-87H cation-exchange column (BioRad Laboratories, Ltd.). Samples were prepared following procedures modified from Marsili et al. [22]. To remove proteins prior to analysis, aliquots of 0.3 mL were placed in microcentrifuge tubes with 0.6 mL acetonitrile and vortexed for about 30 s. Samples were then centrifuged at 10,000×*g* for 10 min. The liquid phase was transferred to HPLC vials using disposable pipettes. Conditions for HPLC were 50 °C, 0.6 mL/min, 0.005 M H_2SO_4 mobile phase, 20-μL injection volume. Peak identities were based on retention times of 1-mg/mL concentration standards (galactose, stachyose, raffinose, fructose, glucose, and sucrose). Sugar concentrations were based on peak heights of standard injections ranging from 5 to 20 μL. Standard solution injections were done in triplicate. Sample concentrations reported are the means of duplicate injections. The concentration confidence intervals reported were calculated based on the uncertainty of the values of the standard curve slope and the y-intercept of the respective saccharide standard curves, as determined by linear regression analysis with the JMP 6.0 statistical software package by SAS, Inc. (Cary, NC, USA).

α-Galactosidase Treatment

Twenty-five microliters of skim fraction was adjusted to pH 6.0 with 1 N HCl in a 50 mL HDPE centrifuge tube. α-Galactosidase (Genencor/Danisco, Rochester, NY, USA) was added to achieve a 1% (w/w, dry basis) concentration. Skim was then incubated at 60 °C for 3 h at 120 rpm in an incubator shaker (Model C24, New Brunswick Scientific, Edison, NJ, USA). This temperature and pH were optimal for the enzyme. After incubating, the pH did not change by more than 0.1 units. The reaction was stopped by adding 20 mL of acetonitrile to 10-mL samples of incubated skim to denature and precipitate all proteins for saccharide analysis.

Results and Discussion

Effects of Enzymes on Extraction of Oil, Protein and Dry Matter

Enzymatic hydrolysis improves oil, protein, and solids extraction during AEP of soybeans [7, 14, 23]. Proteases improve AEP extraction of extruded soybean flakes; however, cellulase treatment does not improve protein and oil extraction yields [14].

The effects of Protex 6L and Protex 7L on oil extraction yield are shown in Fig. 3a. The use of Protex 7L (0.5%) and Protex 6L (0.5 and 1.0%) gave oil extraction yields of 93 and 96–97%, respectively. Freitas et al. [24] achieved 88% of oil extraction yield by using consecutive treatments of cellulase (3%) and protease (3%) during 6 h (3 h for each enzyme) in the EAEP of unflaked and extruded soybeans. Similar oil extraction yield (88%) was reported by Lamsal et al. [14] when using Protex 7L (0.5%) in EAEP of

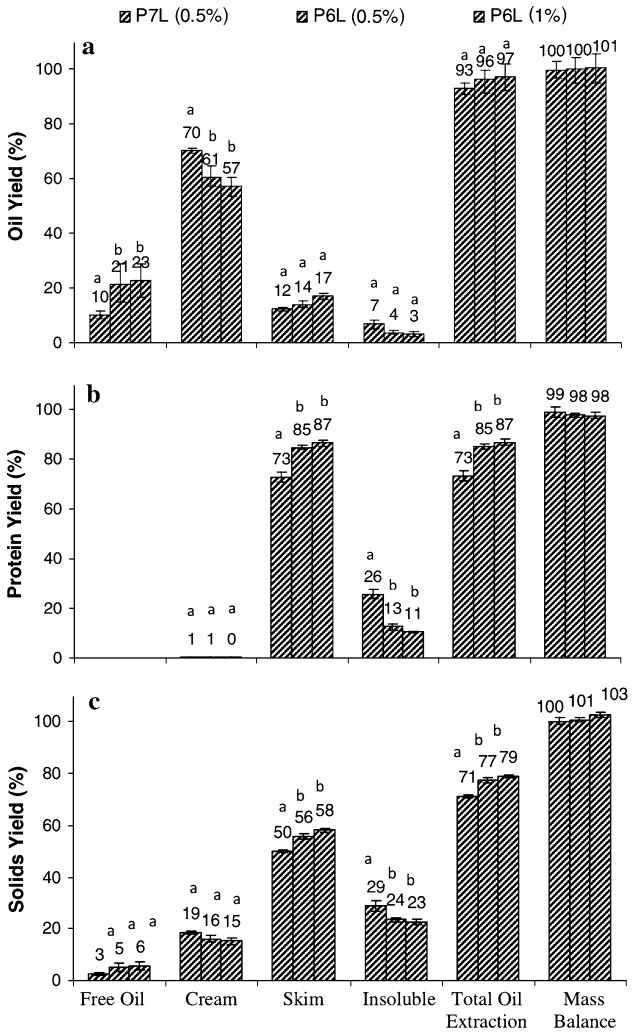

Fig. 3 **a** Oil extraction yield during enzyme-assisted aqueous extraction processing of extruded soybean flakes. **b** Protein extraction yield during enzyme-assisted aqueous extraction processing of extruded soybean flakes. **c** Solids extraction yield during enzyme-assisted aqueous extraction processing of extruded soybean flakes. *P7L*, Protex 7L; *P6L*, Protex 6L

extruded soybean flakes. We achieved higher oil extraction yields (93–97%) than Freitas et al. [24] and Lamsal et al. [14], which we attribute to better selection of enzymes, differences in extruder operation, and soybean variety, age, and storage conditions. Although the mean total oil extractions were not statistically different, more free oil was obtained when using Protex 6L than when using Protex 7L, at both concentrations tested. The yield of free oil was statistically different ($P < 0.05$) when using Protex 6L and Protex 7L at 0.5% dosage. The use of 0.5% Protex 6L yielded twice the amount of free oil obtained with 0.5 % Protex 7L.

Figure 3b shows the effects of using Protex 6L and Protex 7L on protein extraction yield. Protein extraction yields of 73 and 85–87% were obtained with Protex 7L

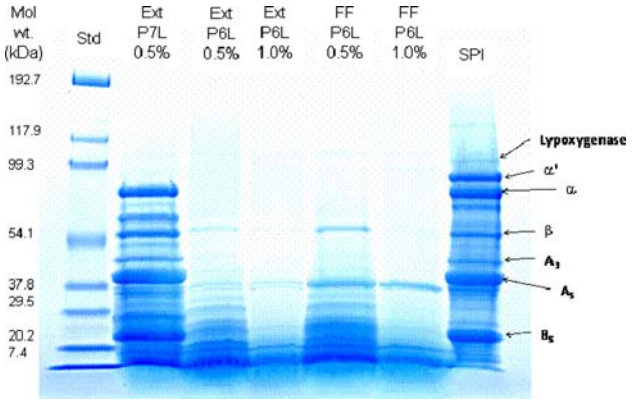

Fig. 4 SDS-PAGE of skim milk after different treatments. *Std*, MW standard; *Ext*, skim from extruded soy flake; *FF*, skim from flaked flour; *SPI*, commercial soy protein isolate, Profam® 646 (Archer Daniels Midland, Decatur, IL, USA). *Letters to the right* indicate specific protein subunits of soybean glycinin and *β*-conglycinin

(0.5%) and Protex 6L (0.5 and 1.0%), respectively. Protein extraction yield of 77% has been reported by Lamsal et al. [14] with Protex 7L (0.5%) in EAEP using extruded soybean flakes. Protex 6L was effective at hydrolyzing more protein than Protex 7L, causing higher protein extraction yields at both concentrations tested. As can be seen in Fig. 4, Protex 6L (0.5%) reduced most peptides to molecular weights of <30 kDa, while Protex 7L (0.5%) yielded peptides with molecular weights of >54.1 kDa. These results agree with Jung et al. [25], where Protex 6L achieved greater extent of hydrolysis with soy flour than Protex 7L.

Except for the protein extracted into the cream, all means were different ($P < 0.05$) when using Protex 6L and Protex 7L at 0.5%. Although increasing the amount of Protex 6L from 0.5 to 1.0% gave the highest degree of hydrolysis with peptides of molecular weight <25 kDa (Fig. 4), significant improvement in the protein extraction yield was not observed. This might indicate that hydrolysis was limited by enzyme selectivity (Fig. 5).

Based on Fig. 3a and b, the conditions that favored protein extraction (0.5% Protex 6L) also favored oil extraction. This trend is in agreement with results reported in the literature [6, 23]. Generally, higher oil extraction occurs with solubilization and/or hydrolysis of protein, which was attributed to the breakdown of the protein network and the oleosin membrane, thereby releasing free oil [3, 6]. Increasing the amount of Protex 6L from 0.5 to 1.0% did not significantly improve oil extraction yield or free oil.

Figure 3c shows the effects of using Protex 6L (0.5 and 1.0%) and Protex 7L (0.5%) on dry matter extraction. Protex 6L was effective at extracting more solids than Protex 7L at both concentrations. Dry matter extraction yields of 71 and 77–79% were achieved when using Protex 7L (0.5%) and Protex 6L (0.5 and 1.0%), respectively. Total dry matter extractions were statistically different at

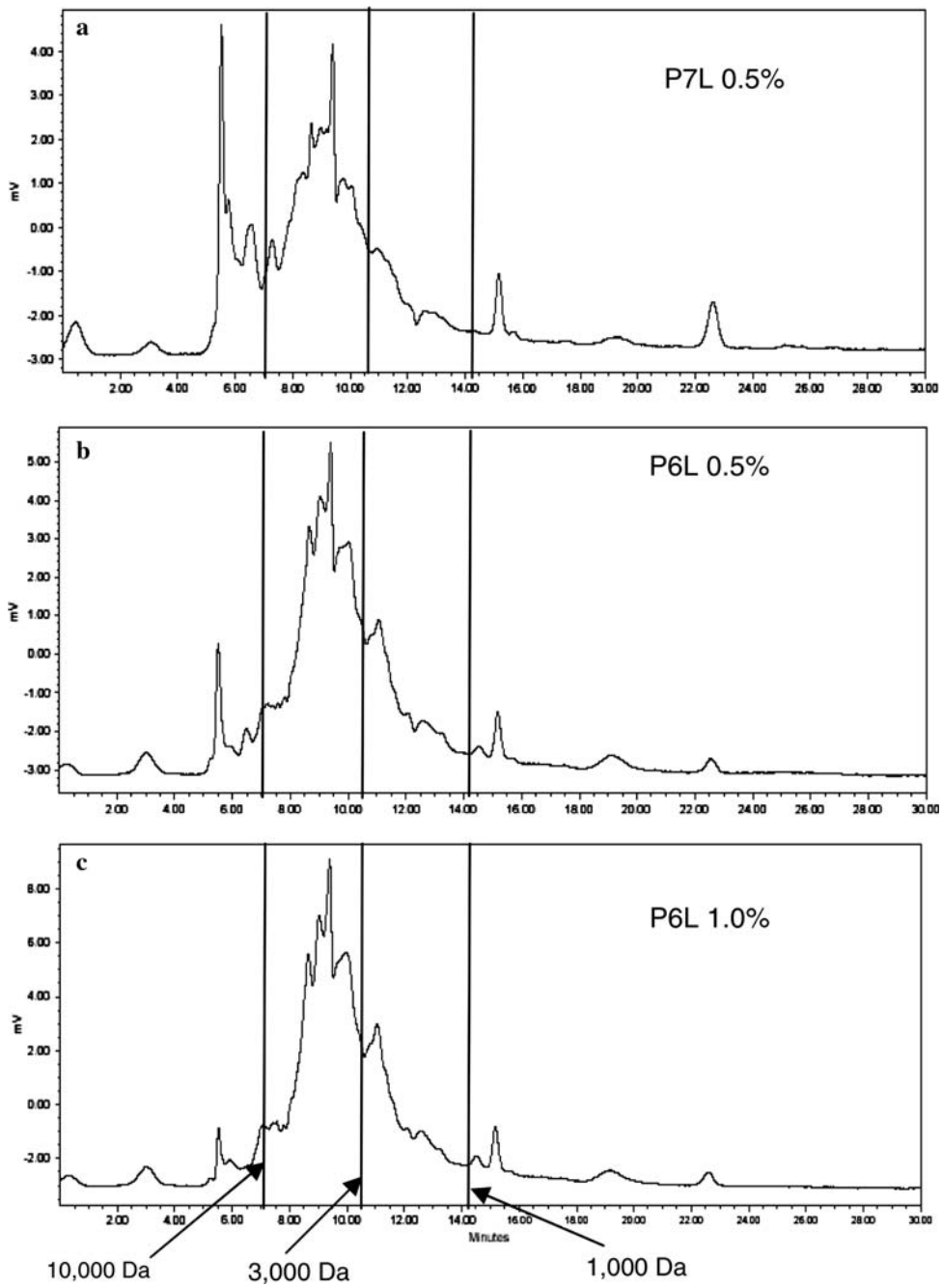

Fig. 5a–c HPLC profiles of the skim fractions of different treatments: **a** 0.5% Protex 7L; **b** 0.5% Protex 6L; **c** 1.0% Protex 6L

$P < 0.05$ when using Protex 6L and Protex 7L at a 0.5% dosage. The higher amount of solids in the skim fraction was consistent with the higher protein extraction yield when using 0.5% Protex 6L. Increasing the amount of Protex 6L from 0.5 to 1.0% did not significantly improve dry matter extraction.

Effects of Enzyme and pH Treatments on Cream De-emulsification

We previously reported that treating Protex 7L cream with 2.5 % LysoMax (phospholipase A2) or 2.5% Protex 51FP as well as pH adjustment to 4.5 with 15 min of stirring at 50 °C yielded 100% free oil [26]. In the present study, enzyme de-emulsification using Protex 6L as well as pH adjustment on Protex 6L and Protex 7L cream were compared. Total destabilization of Protex 6L cream was obtained upon addition of 2.5% Protex 6L (Table 1). When the same conditions were applied to Protex 7L cream, only 91% free oil yield was obtained. Protex 6L cream was also totally destabilized by adjusting the pH to 4.5 and stirring for 15 min at 50 °C. Total destabilization of Protex 6L cream was obtained after adjusting the pH to 4.5 at 25 °C without additional stirring. These results indicated that

Table 1 Effect of extraction and de-emulsification conditions on free oil yield

Extraction conditions	De-emulsification conditions	Free oil yield (%)
0.5% Protex 7L	2.5% Protex 6 [a]	91 a
0.5% Protex 6L	2.5% Protex 6L[a]	100 b
0.5% Protex 6L	pH 4.5, 50 °C, 15 min	103 b
0.5% Protex 6L	pH 4.5, 25 °C, 15 min	101 b
0.5% Protex 6L	pH 4.5, 25 °C, 2 min	100 b
0.5% Protex 6L	pH 4.5, 25 °C, no stirring	100 b
LSD		4.49

Mean values followed by different letters are statistically different ($P < 0.05$)

[a] Reaction was carried out at 50 °C and 90 min

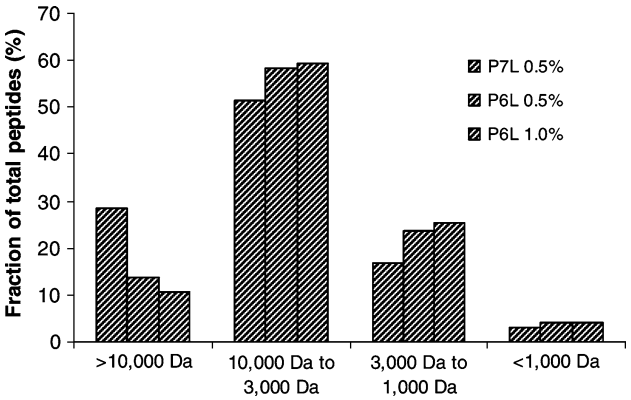

Fig. 6 MW distributions of skim samples based on the peak area of HPLC profiles (means of duplicate injections; duplicate determinations did not differ by more than 1.5%)

Protex 6L cream was more easily destabilized with enzyme treatment and pH adjustment than Protex 7L cream. Using Protex 6L and Protex 7L during extraction affected cream composition (Fig. 3a). When 0.5% Protex 6L was used during extraction, the cream had lower oil content than did Protex 7L cream. In addition, Protex 6L was more aggressive than Protex 7L (see data on skim provided below), and therefore probably generated smaller peptides than did Protex 7L. While more investigation of the peptide profile located at the emulsion interface is needed, we hypothesize that the difference in enzyme aggressiveness and cream composition may contribute to the stability of the cream emulsion.

Polypeptide MW Distribution in Skim

SDS-PAGE profiles of the peptides in the skim fractions of the three protease extraction treatments using extruded flakes are shown in Fig. 4. The two main fractions are β-conglycinin, composed of α', α, and β subunits, and glycinin, composed of A (acidic) and B (basic) subunits. All protease treatments decreased MWs compared to the peptide profile for commercial soy protein isolate. Protex 7L destroyed lipoxygenase and the α' subunit of β-conglycinin. Most other subunits were present in amounts sufficient to stain strongly after Protex 7L treatment. Protex 6L achieved noticeably greater hydrolysis than Protex 7L, reducing most of the peptides to MW <25 kDa. The β subunit of β-conglycinin was more resistant to hydrolysis than other protein subunits. β-Conglycinin has been identified as a potential allergen [16]. The 1.0% Protex 6L treatment completely hydrolyzed all subunits.

MWs of Skim Peptides

Even though the SDS-PAGE gel showed many intact protein subunits after the Protex 7L 0.5 treatment, the SEC profiles (Fig. 5) indicated that a substantial fraction of the proteins was hydrolyzed. The large peak on the left of Fig. 5 represents all proteins of molecular mass greater than about 40 kDa. The area of this peak was very small compared to the area of the profile below 10 kDa. At the same concentration, Protex 6L achieved a greater degree of hydrolysis than Protex 7L. When increasing the concentration of Protex 6L, the large MW polypeptides decreased while the intermediate polypeptides increased, and the small MW polypeptides remained approximately constant. This indicated that the hydrolysis may have been approaching a limit dictated by enzyme selectivity.

The profile areas between the indicated markers are shown in Fig. 6 for a quantitative analysis of the profiles. Most of the polypeptides had MWs between 3 and 10 kDa, with <30% below 3 kDa and <5% below 1 kDa. While TIA reduction and allergenic protein destruction are beneficial, the benefit associated with just the reduction of polypeptide molecular mass in feed applications is not well established. The results of one study indicated that hydrolysis of soy protein resulted in improved weight gain in early-weaned pigs, but the effects of increasing the extent of hydrolysis were not clearly established [18]. In two other studies, Caine et al. [27, 28] concluded that proteolytic treatment does not improve protein digestibility, although no measure of degree of hydrolysis was made in these investigations. Different protease treatment methods as well as a lack of reporting the degree of hydrolysis make it difficult to compare studies on the effects of protein hydrolysis on digestibility.

TIA in Skim

The TIA of skim from extruded soybean flakes was the same as that from unextruded soybean flakes (Fig. 7). This seems to disagree with the generally accepted notion that extrusion destroys TIA [29], even though we used

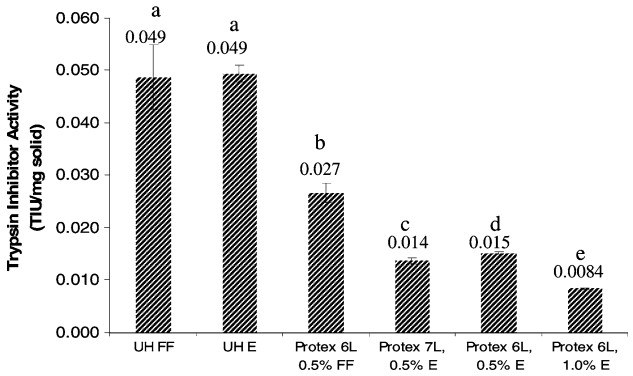

Fig. 7 Trypsin inhibitor activity of skim milk per dry solids basis from extractions of different conditions and starting material: *UH*, unhydrolyzed skim; *FF*, skim from flaked flour; *E*, skim from extruded soy. *Letters* denote statistical differences ($P < 0.05$)

relatively low extrusion temperatures (100 °C). TIA is typically reported per mass of starting material. Indeed, on a per-mass of starting material basis, the extruded material had significantly less TIA than soy flakes (data not shown). In this case, since the product of interest is not the starting material, but rather the extract itself, we reported the activity of the extract only.

Hydrolysis, on the other hand, substantially reduces TIA, and extrusion enhanced this effect. Hydrolysis of extruded soybean flakes Protex 6L reduced TIA by 69%, compared to a 45% reduction by the same treatment of the ground soybean flakes. This suggested that extrusion facilitates hydrolysis of TI. The differences in effects of different enzyme treatments on extruded flakes were less pronounced. The effects of Protex 6L and Protex 7L at 0.5% concentrations were not statistically different, with a 69% reduction in TIA, while Protex 6L at 1.0% achieved 83% reduction. This trend may be a result of the nature of TI in soybean. The two major TIs in soybeans are the Kunitz type, which has a MW of about 20 kDa, and the Bowman–Birk type, which has a MW of 6–10 kDa [16]. As seen in the MW distributions above, most of the polypeptides after hydrolysis were <10 kDa, indicating that the Kunitz-type inhibitor was eliminated, and that the Bowman–Birk inhibitor was responsible for the remaining TIA.

Amino Acid Composition of Skim Protein

The amino acid compositions of the skim fractions are shown in Table 2. The essential amino acid profile of each treatment was not substantially different from the soy protein amino acid profiles reported in the literature. Soy protein is deficient in methionine, but has more lysine than cereal proteins [30]. High temperatures during extrusion can reduce lysine by Maillard reactions, but this did not appear to happen, probably because of the relatively low temperatures used during our extrusion compared to other extrusion studies [31]. Therefore, the amino acid profile of the proteins was not altered by either extrusion or enzyme hydrolysis.

Table 2 Relative amino acid compositions of skim from different treatments

Amino acid	Enzyme used in extraction			Soybean protein (%) [30]
	P7L 0.5 (%)	P6L 0.5 (%)	P6L 1.0 (%)	
Aspartic acid	12.00	11.87	11.81	11.42
Threonine[a]	3.75	4.02	4.03	4.09
Serine	4.51	4.68	4.68	5.39
Glutamic acid	20.19	18.05	17.95	19.30
Proline	4.96	4.95	4.93	5.75
Glycine	4.11	4.22	4.24	4.25
Alanine	4.15	4.42	4.46	4.27
Cysteine	1.54	1.60	1.56	1.43
Valine[a]	4.58	4.84	4.91	4.45
Methionine[a]	1.46	1.56	1.54	1.43
Isoleucine[a]	4.56	4.71	4.75	4.51
Leucine[a]	7.34	7.85	7.92	8.03
Tyrosine	3.67	3.80	3.78	3.85
Phenylalanine[a]	4.91	5.04	5.08	4.82
Lysine[a]	6.85	6.88	6.88	6.34
Histidine[a]	2.62	2.70	2.70	2.60
Arginine[a]	7.30	7.21	7.15	7.33
Tryptophan[a]	1.37	1.41	1.49	0.73
Total	99.9	99.8	99.8	100.0

[a] Essential amino acid

Table 3 Saccharide concentrations of the skim fraction before and after hydrolase treatment

Skim treatment	Stachyose (mg/mL)	Glucose (mg/mL)
P7L 0.5%	3.6 ± 0.5	ND
P7L 0.5% + 1%(w/w) α-galactosidase	ND	3.3 (±0.3)
P6L 0.5%	3.8 ± 0.5	ND
P6L 0.5% + 1%(w/w) α-galactosidase	ND	3.4 (±0.3)
P6L 1.0%	3.3 ± 0.5	ND
P6L 1.0% + 1%(w/w) α-galactosidase	ND	3.9(±0.3)

±95% Confidence interval

ND, not detected

Raffinose and Stachyose Concentrations and Reductions

The HPLC profile for oligosaccharide determination (chromatogram not shown) showed a peak at 6.9 min, matching the elution time for the stachyose standard, with no observable peak at the raffinose elution time. Stachyose is typically present in greater concentrations than raffinose in soybeans [32]. As would be expected, the protease treatments did not affect the stachyose levels of the extracts. Typical sucrose, stachyose, and raffinose levels in whole soybean are 4.1, 3.7, and 1.1%, respectively [32]. Assuming 100% extraction of saccharides, a 1:10 solids-to-liquid ratio would give a stachyose concentration of 3.7 mg/mL, which was in good agreement with the concentrations reported in Table 3. Sucrose inversion occurs on passage through this ion-exchange resin, resulting in two peaks at 9.1 and 9.6 min. While the presence of sucrose was confirmed, the sucrose concentration could not be reliably quantified because of inversion.

Since stachyose is a tetrasaccharide of two galactoses, one glucose, and one fructose, hydrolysis of the glycosidic bonds of the galactose would result in free galactose and sucrose as products. α-Galactosidase enzymes are also known to actively hydrolyze the one to two glycosidic bonds of sucrose, which appeared to be the case in the present study. The HPLC profile, after α-galactosidase treatment, showed that stachyose and sucrose disappeared, while two new peaks appeared, corresponding to glucose and fructose/galactose. Fructose and galactose retention times of 9.97 min and 9.82 min, respectively, were not resolvable. Glucose concentrations based on these results are also shown in Table 3. Glucose is present in stachyose in a 1:1 stoichiometric ratio, so the mass of glucose detected after treatment is about four times greater than would be expected on a mass ratio basis. The extra glucose was probably a result of the hydrolysis of initial sucrose.

Conclusions

Protex 6L was more effective than Protex 7L in extracting more free oil, protein, and solids from extruded soybean flakes during EAEP. Oil and protein extraction yields of 96 and 85%, respectively, were obtained using 0.5% Protex 6L. Increasing the amount of Protex 6L from 0.5 to 1.0% did not significantly improve the oil, protein and dry matter extraction yields. Extracted oil was distributed as 21–23% in the free oil, 57–61% in the cream, and 14–17% in the skim. Although the oil present in the skim fraction was considered extracted from the insolubles, it remained unrecovered as free oil. The cream obtained by EAEP was de-emulsified by enzyme or pH treatment. Using 2.5% Protex 6L totally de-emulsified the Protex 6L cream. The pH treatment was equally efficient at de-emulsifying the cream, as was the enzyme treatment. Total de-emulsification of the Protex 6L cream was obtained without additional stirring after pH adjustment. Enzyme treatment reduced TIA by >80% for extruded soybean flakes. Protein hydrolysis appeared to be limited by enzyme selectivity, with most of the polypeptides being between 1 and 10 kDa for Protex 6L treatments. Raffinose was not present at levels detectable by the assay used, while stachyose was effectively eliminated by α-galactosidase treatment.

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