

Lunasin and Bowman-Birk Protease Inhibitor Concentrations of Protein Extracts from Enzyme-Assisted Aqueous Extraction of Soybeans

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ABSTRACT: Lunasin and Bowman-Birk protease inhibitor (BBI) are two soybean peptides to which health-promoting properties have been attributed. Concentrations of these peptides were determined in skim fractions produced by enzyme-assisted aqueous extraction processing (EAEP) of extruded full-fat soybean flakes (an alternative to extracting oil from soybeans with hexane) and compared with similar extracts from hexane-defatted soybean meal. Oil and protein were extracted by using countercurrent two-stage EAEP of soybeans at 1:6 solids-to-liquid ratio, 50 °C, pH 9.0, and 120 rpm for 1 h. Protein-rich skim fractions were produced from extruded full-fat soybean flakes using different enzyme strategies in EAEP: 0.5% protease (wt/g extruded flakes) used in both extraction stages; 0.5% protease used only in the second extraction stage; no enzyme used in either extraction stage. Countercurrent two-stage protein extraction of air-desolventized, hexane-defatted soybean flakes was used as a control. Protein extraction yields increased from 66% to 89–96% when using countercurrent two-stage EAEP with extruded full-fat flakes compared to 85% when using countercurrent two-stage protein extraction of air-desolventized, hexane-defatted soybean flakes. Extruding full-fat soybean flakes reduced BBI activity. Enzymatic hydrolysis reduced BBI contents of EAEP skims. Lunasin, however, was more resistant to both enzymatic hydrolysis and heat denaturation. Although using enzymes in both EAEP extraction stages yielded the highest protein and oil extractions, reducing enzyme use to only the second stage preserved much of the BBI and Lunasin.

KEYWORDS: lunasin, Bowman-Birk inhibitor, soybeans, enzyme-assisted aqueous extraction

INTRODUCTION

The growing demand for high-protein meal to feed livestock as well as oil for food and biodiesel increased world soybean production to 240 million mt in 2010.¹ Although the primary goal of soybean processing is to produce animal feed protein, ~20% of the soybean is a valuable oil coproduct² that is commonly extracted by direct hexane extraction.³ Increasingly restrictive environmental regulations and health concerns regarding hexane have led to interest in using an aqueous medium to extract oil and protein from many oil-bearing seeds.^{4–11} Enzyme-assisted aqueous extraction processing (EAEP) is an environmentally friendly technology where oil and protein are simultaneously extracted from soybeans.^{5,12–17} In addition to replacing the use of hazardous and polluting hexane, this clean water- and enzyme-based technology enables fractionating soybeans into products suitable for food, feed and fuel.

Oil and protein extracted during EAEP of soybeans are distributed among three fractions including insoluble fiber, skim (protein- and sugar-rich aqueous phase), and an oil-rich cream emulsion. Extractability and recovery in the EAEP of soybeans have been improved by adopting flaking and extrusion,^{12,13} better enzyme selection, enzyme-catalyzed cream de-emulsification,^{14,15} and countercurrent two-stage EAEP instead of one-stage extraction.^{16,17} Oil extractability in countercurrent two-stage

EAEP is 95–99%^{15–17} and is as complete as commercial hexane extraction (95.0–97.5%);³ however, overall free oil recovery is ~83% compared to >95% for hexane extraction due to unrecovered oil in the skim fraction (~14%).¹⁵

Asian populations consuming large amounts of soybean products have lower risks of osteoporosis and some chronic diseases, most notably heart disease and cancer.¹⁸ An array of cancer-preventive phytochemicals has been identified in soy foods.^{19–21} Soy proteins and peptides are receiving considerable attention as promising anticancer compounds. Bowman-Birk protease inhibitor (BBI) is a polypeptide capable of suppressing carcinogenic processes in both in vitro and in vivo animal model systems.²⁰ As a result, the U.S. Food and Drug Administration designated BBI concentrate (BBIC) as an “investigational new drug” in 1992, and is being evaluated in large-scale human trials as an anti-carcinogenic agent.

Lunasin is a peptide in soybeans²² shown to prevent trans-formation of mammalian cells induced by chemical carcinogens and viral oncogenes.^{23,24} Lunasin inhibits cell proliferation arresting

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cell cycle and induces apoptosis in breast, colon, and leukemia cancer cells.^{25–27} When topically administered, lunasin reduced tumor incidence and multiplicity in a skin cancer mouse model.²³ Lunasin also reduces breast tumor incidence and generation in a mouse model.²⁸ Lunasin is the main bioactive component of the BBIC, whereas BBI only protects lunasin from gastrointestinal digestion making it bioavailable to exert anticancer properties. BBI and lunasin have been identified in soy milk, infant formula, tofu, bean curd, and fermented soybean products.²⁹

We recently evaluated the effects of different enzymatic treatments to extract oil and protein from extruded full-fat flakes by using countercurrent two-stage EAEP.³⁰ Maximizing oil and protein recoveries is essential to improving economic viability of EAEP with soybeans; however, different protein extraction yields were associated with changes in protein functionality.³¹ Protein solubility, rate of foaming, and foam stability increased with more extensive hydrolysis while emulsification capacity and stability were reduced. The amino acid composition and in vitro protein digestibilities of the protein were not adversely affected by extrusion or extraction.³¹ Although mechanical and enzymatic treatments enhance extraction yields and protein functionality, the effects of these treatments on important biologically active compounds present in the skim fractions produced by the EAEP of soybeans are unknown. The aim of the present work was to understand the effects of different enzyme usage strategies in countercurrent two-stage EAEP on the lunasin and BBI contents of the skim and to compare them with countercurrent two-stage protein extraction as is used in producing soy protein isolate.

MATERIALS AND METHODS

Soybean Preparation. Full-fat soybean flakes were prepared from variety 92M91-N201 soybeans (Pioneer, a DuPont Business, Johnston, IA) 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) and the hulls were removed from the meats (cotyledons) by aspirating the beans with a multiaspirator (Kice Metal, Wichita, KS). The meats were conditioned at 60 °C by using a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH) and flaked to approximately 0.25 mm thickness using a smooth-surface roller mill (Roskamp Mfg, Inc., Waterloo, IA).

Extruding 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). The moistened full-fat soybean flakes were extruded using a twin-screw extruder (ZSE 27-mm diameter twin-screw extruder; American Leistritz Extruders, Somerville, NJ). High-shear geometry screws were used in corotational orientation at 90 rpm screw speed. The extruder barrel (1080 mm length) was composed of 10 heating blocks operated to achieve a 30–70–100–100–100–100–100–100–100 °C temperature profile. The extruder was manually fed to achieve 10.5 kg/h output rate of extruded flakes. On the basis of our previous results,¹⁷ the flakes were not collected in water. The collets were cooled to room temperature, placed in polyethylene bags, and stored at 4 °C until extracted. The extruded full-fat flakes contained 20.7 ± 1.5% oil (as is), 35.7 ± 0.5% protein (as is), and 11.3 ± 1.0% moisture.

Defatted Flakes. A laboratory percolation extractor-simulator was used to extract oil with hexanes at 50 °C from full-fat soybean flakes. Hexane was added to the flakes at 2:1 (w/w) ratio and five

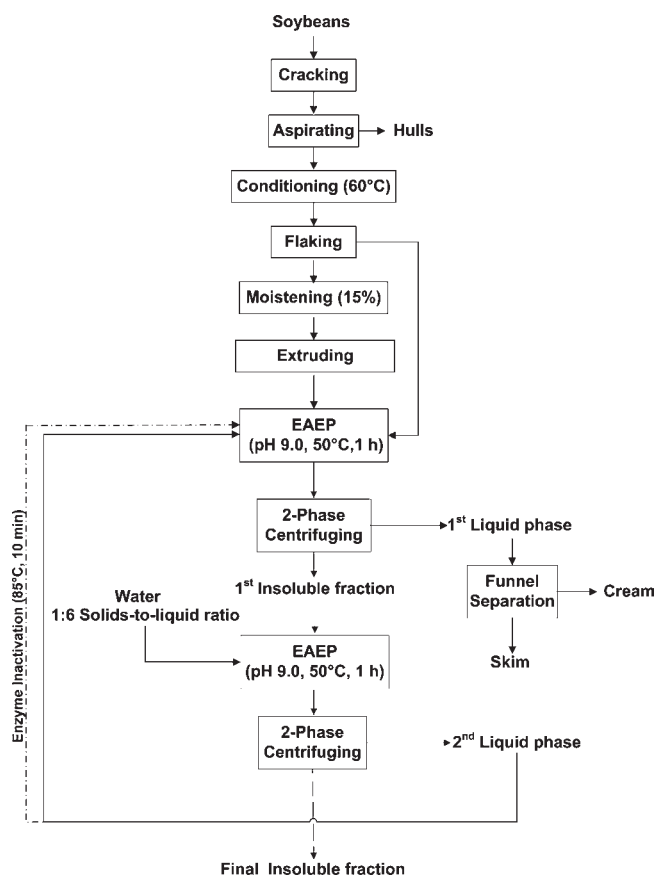


Figure 1. Process flow diagram for the countercurrent two-stage EAEP using different enzyme strategies with extruded full-fat flakes and countercurrent two-stage protein extraction from air-desolventized, hexane-defatted soybean flakes.

extraction stages were used at 6 min/stage followed by 3 min draining/stage. Fresh solvent was used in each extraction stage. Defatted flakes were air-desolventized, placed in polyethylene bags, and stored at 4 °C until used to extract protein.

Enzyme Treatment. Protex 6 L, having 580 000 DU/g minimum activity, was obtained from Genecor Division of Danisco (Rochester, NY) and used in EAEP. Protex 6 L is a bacterial alkaline endoprotease derived from a strain of *Bacillus licheniformis* and has highest activity at pH 7.0 to 10.0 and 30 to 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.¹⁴

Countercurrent Two-Stage EAEP. The extruded flakes were subjected to countercurrent two-stage extraction in a 20-L jacketed glass reactor where the second liquid phase (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) (Figure 1). On the first day of EAEP extraction, the first extraction stage was performed with 1 kg of extruded flakes using 1:6 solids-to-liquid ratio. The slurry pH was adjusted to 9.0 before adding 0.5% Protex 6 L (wt/extruded flakes) and stirred for 1 h at 120 rpm and 50 °C. The slurry obtained in the first extraction stage was centrifuged at 3000 × g to remove the insoluble fiber-rich fraction. The first liquid phase (skim, cream, and free oil) was then placed in a 5-L jacketed reactor and allowed to settle overnight at 4 °C. After settling, the first liquid phase was separated into three fractions (skim, cream,

and free oil). The insoluble fraction obtained from the first extraction stage (1st insoluble) was then subjected to a second extraction stage. Prior to the second extraction stage, the first insoluble fraction was dispersed in water to obtain 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 final insoluble and second liquid phase.

The second liquid phase was recycled to the first extraction stage on the next trial in two different ways: (1) without any heat treatment and therefore had active enzyme activity in both stages (treatment 1); or (2) heated for 10 min at 85 °C to inactivate the enzyme prior to the first stage of countercurrent extraction (treatment 2). The same extraction procedure was performed, but without enzyme in either extraction stage with extruded flakes (treatment 3) and with air-desolventized, hexane-defatted soybean flakes (treatment 4). The extractions in the second, third, and fourth trials were performed in the same manner as the first trial. Since steady-state extraction was achieved after the second extraction trial,¹⁷ samples from the third and fourth extraction trials were analyzed to determine chemical compositions and mass balances of oil, protein, and solids.

Freeze-Drying EAEP Skim Fractions. The skim fractions from two different extraction batches were frozen at -14 °C for at least 24 h and placed in a Virtis Ultra 35 (Gardiner, NY) freeze-dryer with shelves cooled to -40 °C. Vacuum was applied while the temperature was held constant until the vacuum dropped to 100 mTorr. Shelf heating was then increased to 26 °C and held constant thereafter. The complete freeze-drying cycle lasted for 72 h. Samples were placed in sealed containers and stored at room temperature until analyzed.

Oil, Protein, and Solids Recoveries. The freeze-dried skims were analyzed for oil, protein, and moisture contents. Total fat contents were determined by using the acid hydrolysis Mojonnier method (AOCS method 922.06), protein contents by using the Dumas combustion method and the N conversion factor of 6.25 (vario MAXCN Elementar Analysensysteme GmbH, Hanau, Germany), and total solids by gravimetric means after drying 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. Chemical analyses were performed in duplicate on freeze-dried samples from two different extraction batches (third and fourth extraction trials).

Size-Exclusion Chromatography of Skim Polypeptides. Low-MW polypeptides were characterized by using a Galaxie controlled Varian HPLC system (Walnut Creek, CA) with a Prostar 410 Autosampler, Prostar 210 Solvent Delivery Module, and a Prostar 325 UV-vis Detector equipped with a Biorad Biosil 400-5 column (300 × 7.8 mm) and a Biorad Biosil 400 Guard, 80 × 7.8 mm guard column up stream. The mobile phase was 0.05 M NaH₂PO₄, 0.05 M Na₂HPO₄, and 0.15 M NaCl at pH 6.8 in 18 MQ water at 1.0 mL/min flow rate and ambient temperature. Samples were dispersed in distilled water at 2.5 mg/mL concentration and 30 μL aliquots were injected. Absorbance was measured at 280 nm. MW markers (Biorad, Berkeley, Ca) were thyroglobulin (670 000), bovine gamma globulin (158 000), chicken ovalbumin (44 000), equine myoglobin (17 000), and vitamin B12 (1350). Freeze-dried samples from two different extraction batches (third and fourth extraction trials) were analyzed in duplicate.

SDS-PAGE. MW profiles of skim peptides were determined by SDS-PAGE on 12% polyacrylamide gels (BioRad). A low-range MW marker (6.5–66 kDa) was used. Each sample was diluted to 1.78 mg/0.5 mL protein concentration in a 2x sample buffer (Urea-SDS-PAGE), heated in boiling water for 5 min, and loaded into the gel well by using 10-μL aliquots for loading 35 μg of protein. After destaining, the gels were scanned with an Amersham Pharmacia Biotech Image Scanner (Piscataway, NJ). Freeze-dried samples from two different extraction batches (third and fourth extraction trials) were analyzed in duplicate.

Lunasin and BBI Analyses. A 500-mg portion of soy samples was added to 10 mL of distilled water and magnetically stirred overnight. The samples were centrifuged at 15 300 ×g for 30 min and the supernatants were collected for lunasin and BBI analysis. The analysis was carried out following the method described by Hernández-Ledesma and co-workers,²⁹ with some modifications. Soy extracts (100 μL), synthetic lunasin (Chengdu Kaijie Bio-Pharmaceutical Co., Chengdu, P.R. China) (33 μM), or commercially prepared BBI (Sigma) (38 μM) were added to 200 μL of tricine sample buffer (Bio-Rad) for lunasin analysis, or 100 μL of Laemmli sample buffer (Bio-Rad) for BBI analysis, and heated at 100 °C for 5 min. After the samples and standard had cooled to room temperature, they were loaded onto 16.5% Tris-tricine polypeptide gels or 15% Tris-HCl gels (Bio-Rad) for lunasin and BBI identification, respectively. The gels were run in Mini Protean-2 Cells (Bio-Rad) by using Tris-tricine-SDS (lunasin analysis) or Tris-glycine (BBI analysis) buffer.

In the case of lunasin analysis, the conditions were set at 100 V constant, and the gels were run for 100 min. For BBI analysis, the conditions were set at 200 V constant, and the gels were run for 40 min. An Immun-Blot PVDF membrane (Bio-Rad) was prepared for transfer by soaking in 100% methanol and rinsing with distilled water. The proteins on SDS-PAGE gel were transblotted to the membrane for 60 min at 100 V and 4 °C. After transferring, the nonspecific binding sites were blocked by immersing the membrane for 1 h in Odyssey Blocking buffer (Li-Cor Biosciences, Lincoln, NE). The membrane was washed with fresh changes of phosphate buffer saline (PBS)-Tween 20 (PBS-T) and incubated with lunasin monoclonal primary antibody (diluted 15:10,000 in Odyssey Blocking buffer-Tween 20) or BBI monoclonal primary antibody (diluted 5:10,000 in Odyssey Blocking buffer-Tween 20) overnight at 4 °C. After washing with PBS-T, the membrane was incubated for 1 h with antimouse IRDyE secondary antibody (Li-Cor Biosciences) at 1:10,000 dilution Odyssey Blocking buffer-Tween 20. After washing the membrane four times with PBS-T and once with PBS, it was developed by using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Lunasin and BBI contents were calculated by comparing the band intensities with those of known lunasin standards run under the same conditions. The intensities of the bands were quantified using *Un-SCAN-IT gel* version 5.1 software (Silk Scientific, Inc., Orem, UT). Freeze-dried samples from two different extraction batches (third and fourth extraction trials) were analyzed in triplicate. Results were expressed as means of the three values.

Statistical Analyses. The experiment was a completely randomized design and 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). Means were compared by using F-protected contrasts at *P* < 0.05 significance level.

Table 1. Effects of Enzyme Treatments on Extraction Yields of Oil, Protein, and Solids in Countercurrent Two-Stage EAEP^{a,b,c,d,e}

treatment	extraction yields (%)		
	oil	protein	solids
treatment 1 ^a	98.8 ^E	96.0 ^E	83.7 ^E
treatment 2 ^b	94.0 ^F	89.1 ^F	79.2 ^F
treatment 3 ^c	83.6 ^G	66.0 ^G	65.6 ^G
treatment 4 ^d	59.6 ^H	85.2 ^F	69.4 ^H

^a Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in both extraction stages. ^b Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in the second extraction stage only. ^c Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes without using protease in either extraction stage. ^d Protein extract from countercurrent 2-stage extraction of air-desolvitized, hexane-defatted soybean flakes without using protease in either extraction stage. ^e Superscript E–H means (two independent extraction batches) within the same column followed by different letters are statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of Extraction Treatments on Yields of Oil, Protein, and Solids. We recently reported the effects of enzyme use on oil, protein, and solids extraction yields from extruded full-fat soybean flakes.^{17,30} Reducing enzyme exposure in EAEP decreased all extraction yields. Extraction yields were greater when using enzyme in both EAEP extraction stages (treatment 1) and moderately greater when using enzyme in the second stage only (treatment 2) compared to no enzyme use (treatment 3). Countercurrent two-stage protein extraction without using enzyme from air-desolvitized, hexane-defatted soybean flakes yielded approximately 85% of the original soy protein (Table 1). The more stages the enzyme was used in EAEP, the more solids solubilized. The lower protein extraction yield from countercurrent two-stage extraction without enzyme of extruded full-fat soybean flakes (Treatment 3) compared to using the same extraction procedure with air-desolvitized, hexane-defatted soybean flakes was attributed to protein denaturation during extrusion (66 vs 85%). Using enzyme during extraction from extruded flakes (Treatments 1 and 2) restored protein solubility thus improving protein extraction yields to 89–96%.

Characterization of Freeze-Dried Skim Fractions Obtained by Different Extraction Treatments. We previously reported that regardless of enzyme treatment used to perform EAEP with extruded full-fat soybean flakes (treatments 1–3), oil contents in the freeze-dried skim fractions were similar; however, decreasing enzyme exposure during extraction yielded skim fractions with lower protein contents.³⁰ The absence of enzyme during extraction of air-desolvitized hexane-defatted flakes (treatment 4) produced extract with similar protein contents to skims obtained by EAEP when using extruded full-fat flakes and enzyme (treatments 1 and 2) as shown in Table 2. Lower protein extractability in countercurrent two-stage protein extraction from air-desolvitized, hexane-defatted soybean flakes (Treatment 4) was counterbalanced by achieving higher protein purity due to absence of fat compared to EAEP using extruded full-fat flakes. More extensive use of enzyme produced higher protein contents in EAEP skims. When not using enzyme, the higher protein content of skim obtained from air-desolvitized, hexane-defatted flakes

Table 2. Fat and Protein Contents of Freeze-Dried Skims and Protein Extracts Obtained by Using Different Enzyme Strategies in EAEP of Soybeans^{a,b,c,d,e}

treatment	fat (% db)	protein (% db)
treatment 1 ^a	6.1 ^E	61.0 ^E
treatment 2 ^b	6.5 ^E	59.7 ^E
treatment 3 ^c	6.1 ^E	57.2 ^F
treatment 4 ^d	1.6 ^F	61.1 ^E

^a Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in both extraction stages. ^b Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in the second extraction stage only. ^c Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes without using protease in either extraction stage. ^d Protein extract from countercurrent 2-stage protein extraction of air-desolvitized, hexane-defatted soybean flakes without using protease in either extraction stage. ^e Superscript E,F mean (two independent extraction batches) within the same column followed by different letters are statistically different at $P < 0.05$.

demonstrated the adverse effect of extrusion on protein extraction (treatment 3).

SDS-PAGE and Size-Exclusion Chromatography (SEC) of Polypeptides. The peptide profiles from the skim fractions of the four extraction treatments are shown in Figure 2. Skim from countercurrent two-stage EAEP using enzyme in both extraction stages (treatment 1) produced peptides with the lowest MW; while using enzyme in the second stage only of countercurrent two-stage EAEP (treatment 2) produced peptides of modestly reduced MW. Using enzyme in both extraction stages reduced the subunits of the two main soybean proteins (β -conglycinin and glycinin) to peptides having MW < 20 kDa. Although SDS-PAGE gels showed similar profiles of intact protein subunits in skim when using enzyme in the second stage only of countercurrent two-stage EAEP with extruded full-fat soybean flakes (treatment 2) compared with skims from treatments 3 and 4 (without enzyme use), the quantitative analysis of the profiles (Figure 3) indicate that peptides with MW > 17 kDa were 20, 32, 43, and 30% for skims from treatments 1, 2, 3, and 4, respectively. Although no enzyme was used in either Treatment 3 or Treatment 4, lower amounts of peptides with WM > 17 kDa were observed in the extracts from air-desolvitized, hexane-defatted soybean flakes (treatment 4). This trend can be observed when comparing peptides with MW < 1350 Da, 47 vs 56%, respectively.

Lunasin and BBI Concentrations in Skim Fractions Obtained by Different Extraction Treatments. SDS-PAGE and Western-blots were used to analyze lunasin and BBI contents of skim fractions obtained by different extraction treatments. Two bands were detected in the Western blots having MWs of 5 and 8 kDa, which correspond to lunasin and BBI, respectively (Figure 4). The calculated concentrations of these peptides (mg/g protein) are shown in Table 3. Lunasin contents were similar for the starting materials, extruded full-fat flakes and air-desolvitized, hexane-defatted flakes. The detection of lunasin in both samples confirms the stability of lunasin to heat as reported by Galvez et al.;²³ however, BBI concentration was substantially reduced by extrusion. Although we used relatively low extrusion temperatures (100 °C), protein denaturation reduced protein solubility and extraction, which could have been responsible for BBI degradation in extruded flakes.

In general, enzymatic hydrolysis reduced both lunasin and BBI concentrations in EAEP skim fractions obtained from extruded

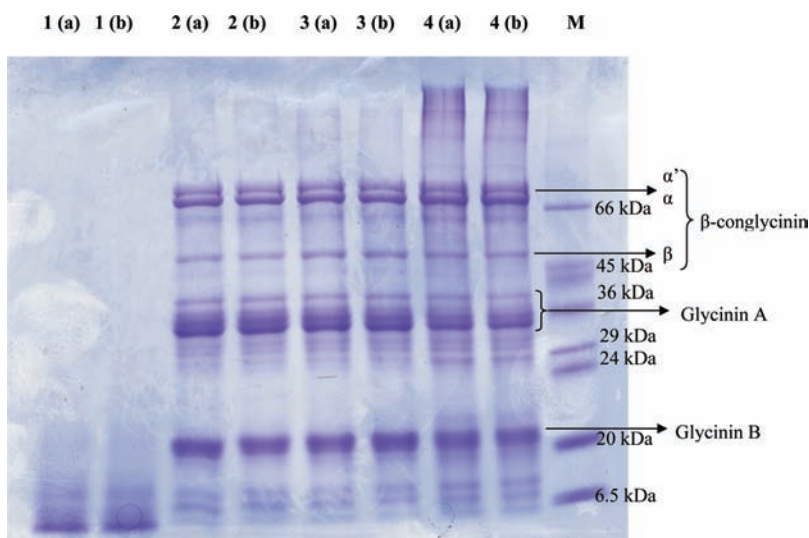


Figure 2. SDS-PAGE gel separation of extracted proteins using different enzyme strategies with extruded full-fat flakes and air-desolventized, hexane-defatted soybean flakes. Treatment 1: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in both extraction stages; treatment 2: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in the second extraction stage only; treatment 3: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes without using protease in either extraction stage; and treatment 4: protein extract from countercurrent 2-stage protein extraction of air-desolventized, hexane-defatted soybean flakes without using protease in either extraction stage. Parts A and B refers to samples from two independent extraction batches.

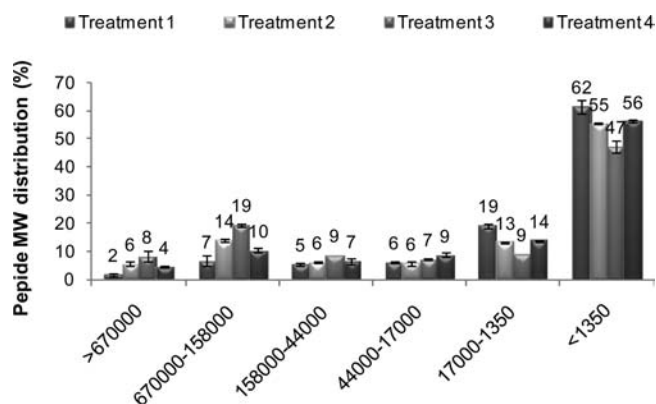


Figure 3. MW distributions of peptides based on the peak area of HPLC profiles. Means and standard deviations are for samples from two independent extraction batches. Treatment 1: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in both extraction stages; treatment 2: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in the second extraction stage only; treatment 3: skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes without using protease in either extraction stage; and treatment 4: protein extract from countercurrent 2-stage protein extraction of air-desolventized, hexane-defatted soybean flakes without using protease in either extraction stage.

full-fat flakes. Reducing enzyme exposure as shown in going from treatment 1 (using enzyme in both extraction stages) to treatment 3 (no enzyme used) increased lunasin content from 6.7 to 8.8 mg/g protein, respectively. This trend was also observed for BBI, but to a greater extent. BBI content increased from 4.0 to 21.5 mg/g protein when reducing enzyme exposure (treatment 1 vs 3). These results are in agreement with Moura et al,¹⁴ who observed that enzyme hydrolysis during EAEP reduced trypsin inhibitors and the reduction was enhanced by extrusion. The skim fraction

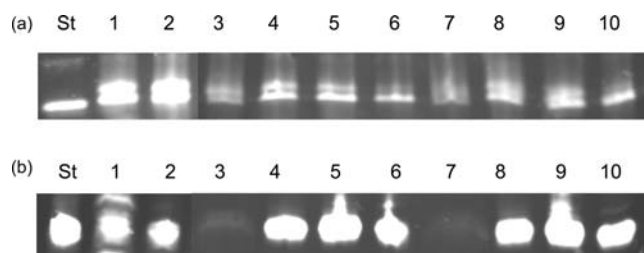


Figure 4. (a) Western-blot analysis of lunasin obtained from enzyme-assisted aqueous extracted soy protein samples. Lane St contains 188 ng of synthetic lunasin; lane 1: extruded full-fat soybean flakes; lane 2: air-desolventized hexane-defatted soybean flakes; lane 3: protein extract 1A; lane 4: protein extract 2A; 5: skim 3A; 6: skim 4A; 7: skim sample 1B; 8: skim sample 2B; 9: skim sample 3B; and 10: skim sample 4B. Each lane contains 30 μ g of protein. (b) Western-Blot analysis of BBI obtained from enzyme-assisted aqueous extracted soy protein samples. Lane St contains 1 μ g of synthetic BBI. 1: Extruded flakes (200 μ g protein); 2: Air-desolventized, hexane-defatted flakes (70 μ g protein); 3: Protein extract 1A (200 μ g protein); 4: Protein extract 2A (75 μ g protein); 5: Skim 3A (75 μ g protein); 6: Skim 4A (50 μ g protein); 7: Skim sample 1B (200 μ g protein); 8: Skim sample 2B (75 μ g protein); 9: Skim sample 3B (75 μ g protein); 10: Extract sample 4B (50 μ g protein). Parts a and b refer to samples from two independent extraction batches.

obtained by countercurrent two-stage extraction of air-desolventized, hexane-defatted flakes (treatment 4) had the highest BBI content (26 mg/g protein). When skim was obtained from extruded full-fat flakes without using enzyme (treatment 3), BBI content was reduced from 26 to 21.5 mg/g protein. Moreover, when using enzyme in the second extraction stage (treatment 2) and in both extraction stages (treatment 1), BBI content significantly decreased to 18.1 and 4.0 mg/g protein, respectively. These results indicate that BBI is sensitive to enzymatic hydrolysis and heat denaturing with small reductions in lunasin contents being observed when increasing exposure to enzyme.

Table 3. Lunasin and Bowman-Birk Protease Inhibitor (BBI) Concentrations of Freeze-Dried Skims and Protein Extracts Obtained by Using Different Enzyme Strategies in EAEP of Soybeans^a
^{b,c,d,e}

sample	lunasin		BBI	
	mg/g protein	g/100 g product (db)	mg/g protein	g/100 g product (db)
extruded full-fat flakes	7.23 ^E	0.30 ^E	5.0 ^E	0.20 ^E
air-desolventized, hexane-defatted flakes	7.09 ^E	0.35 ^F	13.0 ^F	0.64 ^F
treatment 1 ^a	6.74 ^E	0.41 ^E	4.0 ^E	0.25 ^E
treatment 2 ^b	7.92 ^F	0.47 ^F	18.1 ^F	1.06 ^F
treatment 3 ^c	8.78 ^G	0.53 ^G	21.5 ^F	1.30 ^F
treatment 4 ^d	7.02 ^E	0.41 ^E	26.1 ^G	1.55 ^G

^a Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in both extraction stages. ^b Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes using protease in the second extraction stage only. ^c Skim from countercurrent 2-stage EAEP of extruded full-fat soybean flakes without using protease in either extraction stage. ^d Protein extract from countercurrent 2-stage protein extraction of air-desolventized, hexane-defatted soybean flakes without using protease in either extraction stage. ^e Superscript E,F,G mean (two independent extraction batches were analyzed in triplicate) within the same column followed by different letters are statistically different at $P < 0.05$.

Using protease enhanced protein, oil and dry matter extraction in countercurrent two-stage EAEP of extruded full-fat soybean flakes. Substantially more protein was extracted with enzyme in countercurrent two-stage EAEP of extruded full-fat soybean flakes than when using countercurrent two-stage protein extraction of air-desolventized, hexane-defatted soybean flakes that simulates protein extraction methods used in preparing soy protein isolate. Fat contents of the dried protein extracts were about four times greater when using extruded full-fat soybean flakes than when using air-desolventized, hexane-defatted soybean flakes. Extrusion and enzymatic hydrolysis significantly reduced BBI contents in skim fractions obtained by using countercurrent two-stage EAEP of soybeans; however, lunasin was more resistant to both enzymatic hydrolysis and heat.

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