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ORIGINAL PAPER

Structure, Protein Interactions and In Vitro Protease Accessibility of Extruded and Pressurized Full-Fat Soybean Flakes

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Abstract The objectives of the present study were to determine how extrusion (barrel temperature of 100 °C) and high-pressure processing (HPP, 200 and 500 MPa, 15 min, 25 °C) of full-fat soybean flakes (FFSF) modified the structure of soybean cotyledon cells, the protein interactions and the in vitro protease accessibility. Cellular disruption of the cotyledon cells was only observed for extruded FFSF. Extrusion and HPP at 500 MPa favored formation of insoluble protein aggregates, in which oil was entrapped. High pressure size exclusion chromatography (HPSEC) and extraction methods using buffers containing SDS and 2-mercaptoethanol suggested that noncovalent interactions were the main forces in protein aggregate formation during HPP 500 MPa and extrusion. Intermolecular cross-linking by disulfide bonding was also involved in insoluble aggregates, but at a lesser extent than noncovalent interactions. Extrusion and HPP 500-MPa treatment enhanced the proteolytic attack, while treatment at 200 MPa had no impact. Drastic changes in the peptide profile of the extracted proteins were, however, only observed for the enzyme-treated 500-MPa FFSF. Optimal oil and protein extraction yields required cellular disruption of cotyledon cells and hydrolysis of protein aggregates, which were obtained with enzyme-assisted aqueous extraction of extruded FFSF.

Keywords Extrusion · High-pressure processing · Soybean oil · Protein

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Introduction

Solvent extraction with *n*-hexane is the traditional process for recovering edible oils from oilseeds. Alternatives are being sought to overcome the major concerns of this process, including the classification of *n*-hexane as a hazardous air pollutant by the US Clean Air Act. Among the potential alternatives available, enzyme-assisted aqueous extraction processing (EAEP) of extruded full-fat soybean flakes (FFSF) is an environmentally friendly substitute that has reawakened a lot of interest mainly because of the high oil extraction yield recently reported (>90%) and some inherent advantages of the process such as the simultaneous extraction of soy proteins [1–4].

Extrusion cooking of isolated soybean proteins promotes formation of noncovalent and disulfide bonding, and their relative proportion is a function of many parameters, including temperature of treatment, moisture content, shear level, and screw configurations [5–9]. The nature of protein interactions that occur during extrusion of soybean flakes are more likely to play an important role in the in vitro protein digestibility [10] taking place during EAEP. The characterization of the nature of these interactions could be useful in the selection of the processing conditions for optimum release of both oil and proteins during aqueous extraction of extruded material.

To identify the importance of cellular disruption and protein changes that occur during extrusion pretreatment of soybean flakes on the subsequent aqueous extraction, the flakes were also pretreated with high-pressure processing (HPP). HPP of food is a non-traditional processing approach, during which pressure up to 600 MPa (87,000 psi) is applied isostatically, spontaneously, and uniformly throughout the product [11]. Under pressure, covalent bonds remain unchanged. The main interactions

found in pressurized soy proteins are hydrophobic, electrostatic, and disulfide linkages [12–14]. Among the processing parameters affecting protein changes most, the pressure level has an important impact on the proportion of protein denaturation and structural configurations induced by the treatment [15].

This study was intended to shed light on the molecular mechanisms occurring during extrusion and high-pressure processing and to establish the differences between high-pressure and extrusion with respect to the structural modifications induced by the treatment and in vitro accessibility toward a protease. In addition, information on the bonding nature of the protein–protein interactions that occurred during extrusion and HPP of full-fat soybean flakes along with molecular size of solubilized proteins was provided. The oil and protein extraction yields recovered after aqueous extraction processing (AEP) and EAEP of extruded and pressurized full-fat soybean flakes were reported in a previous study [16].

Materials and Methods

Full-Fat Soybean Flakes Preparation

FFSF was prepared at the Center for Crops Utilization Research at Iowa State University from variety 92M91 soybeans harvested in 2006 in Iowa, USA. The soybeans were cracked in a roller mill (Model 10X 12SGL, Ferrell-Ross, Oklahoma, OK) and aspirated using a cascade aspirator (Kice Metal, Wichita, KS, USA) to separate the hulls. The dehulled soybeans were conditioned to 60 °C using a triple-deck seed conditioner (French Oil Mill Machinery Co., Piqua, OH, USA) and were flaked using a smooth-surfaced roller mill (Roskamp Mfg Inc., Waterloo, IA, USA) to approximately 0.30 mm thickness. The flakes were sealed and stored in plastic bags at 4 °C until used. Before extrusion and HPP, the soy flakes were adjusted to a moisture level of ~12–15% with a Gilson mixer (Model 59016A, St. Joseph, MO, USA). The moisture-adjusted flakes were then placed into double polyethylene bags and kept at 4 °C until used. These flakes contained 19% oil (dry basis) and 32% crude protein (dry basis).

Extrusion

The extrusion was carried out on a Micro ZSE-27 twin-screw extruder (American Leistritz Extruders, Somerville, NJ, USA; Fig. 1). The unit was equipped with a 4-mm diameter die. The length and diameter of each screw was 1,080 and 27 mm, respectively. The screw configuration used in the experiments consisted of conveying elements (Length/Diameter (L/D) = 8.0), kneading element (L/D = 5.4), conveying element (L/D = 4.6), kneading element (L/D = 3.4), conveying element (L/D = 4.6), kneading element (L/D = 3.4), conveying element (L/D = 2.2), kneading element (L/D = 2.2), and a conveying element (L/D = 8.0). The barrel consisted of ten independently controlled heating barrels. The barrels also had jackets in which air was circulated at controlled flow rates via solenoid valves to achieve consistent temperatures during processing. The temperatures of each of the barrels, measured via Fe-CuNi thermo elements inserted in the bottom of each barrel, during the process were: 30 °C for the feed barrel, 70 °C for barrel 1, 100 °C for barrels 2–9. Soy flakes were fed into the unit by hand at a rate of 18.2 kg/h and processed via the intermeshing co-rotating screw at constant rpm of 90. The output under these

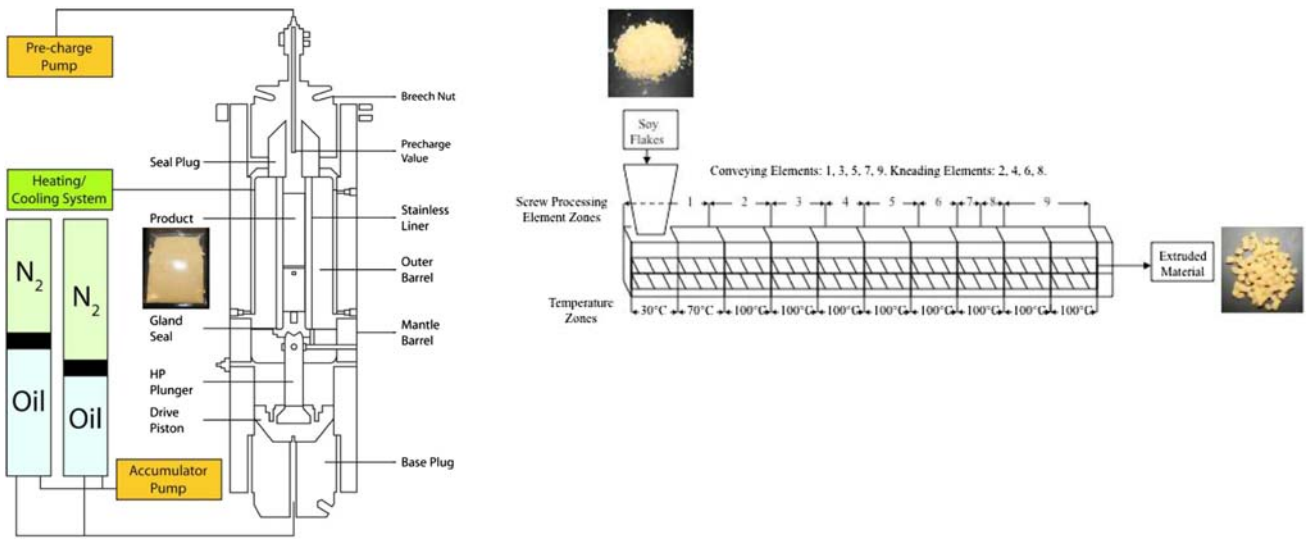

Fig. 1 Schematic of the high pressure vessel and double screw extruder

conditions was 11 kg/h of extruded soy flakes and the residence time of the material was 1 min. Processed material was fed through until equilibrium conditions were reached before material was collected for experimental use. Moisture content of the extruded material was between 9 and 10%.

High-pressure Processing

One hundred grams of the prepared soy flakes and 300 g of distilled water (1:3 flake-to-water ratio) were placed in a polyester bag (Sealpaks, KAPAK, Minneapolis, MN, USA) and the pack was sealed such that the headspace in the pouch was kept to a minimum. The samples were pressurized at 200 and 500 MPa at an initial temperature of 25 °C for a dwell time of 15 min using a Food-Lab 900 High-Pressure Food Processor (Stansted Fluid Power Ltd, Stansted, UK; Fig. 1). The sample holder had an internal diameter of 6.5 cm and was 23 cm high. The rates of pressurization and depressurization were 260 and 500 MPa/min, respectively. The pressurization fluid was a 1:1 mixture of 1,2 propanediol and water (GWT Global Water Technology, Inc., Oakbrook Terrace, IL). The temperature increase of the pressurization fluid due to adiabatic heating was ~3 °C/100 MPa. Each treatment was conducted independently in triplicate.

Aqueous Extraction and Determination of Degree of Hydrolysis

Extractions were conducted at a final flake-to-water ratio of 1:10 in a 4-L jacketed reactor (Chemglass, Vineland, NJ, USA) at 380 rpm. With flakes and extruded flakes, the 1:10 ratio was based on the weight of the starting material (as is). For the pressurized samples, 7 volumes of water were added to the pressurized slurry to reach the 1:10 ratio. After 1 h of reaction at 50 °C and pH 7.0, the pH was raised to 8.0 with 2 N NaOH and the extraction was carried out for 15 min. This procedure was referred to as aqueous extraction process (AEP). For the EAEP, Protex 7L was added at a dose of 0.5% (w/w, on the basis of starting material, as is). Protex 7L (EC 3.4.21.62 and EC 3.4.24.38) is a bacterial neutral protease preparation with mainly endopeptidase activities derived from the controlled fermentation of a nongenetically modified strain of *Bacillus amyloliquefaciens*. This enzyme was kindly provided by the Genencor Division of Danisco (Rochester, NY). Its optimum pH varied between 6.0 and 8.0, and optimum temperature between 40 and 60 °C (data provided by Genencor). During AEP and EAEP, the pH was maintained at a constant value with the addition of 2 N NaOH using a pH-stat (718 Stat Titrino, Methrom, Brinkmann Instruments Inc., Westbury, NY, USA). The degree of hydrolysis (DH) was determined during the 1-h reaction at pH 8.0 as described by Jung et al. [17]. Separation of the liquid and insoluble residue was carried out by centrifugation with a JS 4.0 swinging bucket rotor (Beckman Coulter, Inc., Fullerton, CA, USA) at $3{,}000\times g$ for 15 min at room temperature. The cream and free oil that floated at the surface was discarded and an aliquot of the skim was used for SDS PAGE.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The skim extract was diluted with 0.5 M Tris–HCl, urea (30%), glycerol (20%), 0.4% bromophenol blue (2.5%), 10% SDS solution (2%) and 2-mercaptoethanol (2%), pH 6.8, and boiled for 5 min. Once cooled down, the samples were centrifuged at $10{,}000\times g$ for 10 min and the supernatant was stored at −30 °C until electrophoresis was run. The resolution gel was a 4–20% gradient, and the stacking gel was 4% (161-1105, Bio-Rad Laboratories, Hercules, CA, USA). The gels were run at 200 V using mini-slabs (Mini-Protean® II model, Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained according to the procedure of Neuhoff et al. [18]. A molecular marker (M3913, Sigma, St Louis, MO, USA) was used to compare and identify the unknown bands from SDS-PAGE gels for which 30 μg of soy protein was loaded per lane.

Microscopic Observations

A 3–5 g aliquot of the insoluble fraction was fixed for 48 h at 4 °C into a 2% paraformaldehyde and 2% glutaraldehyde fixative 0.1 M cacodylate buffer, pH 7.2. Two milliliters of this slurry was centrifuged at 1,000 rpm for 3 min and washed four times for 10 min. The material was then fixed with 1% osmium tetroxide for 1 h at room temperature. The samples were centrifuged as previously mentioned and washed with deionized water two times for 10 min. The material was dehydrated through a graded ethanol series. The samples were further dehydrated with ultrapure acetone and infiltrated with Spurr's epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA) and embedded and polymerized at 65 °C for 48 h. Sections of 1 μm thick were made using a Leica UC6 Ultramicrotome, and stained with 1% toluidine blue. Images were taken using the Zeiss Axioplan II light microscope with an MRC digital camera and Axiovision software.

Solubility in Extracting Solvents

An aliquot of 0.5 g of the extruded FFSF was dispersed in 10 mL of selected buffer for 2 h at 30 °C. For pressurized samples, 0.5 g of FFSF was dispersed in 10 mL of the

buffer and then pressurized to the selected conditions prior to the 2 h-stirring step. The slurries were centrifuged at 8,000×g, 10 min, 20 °C, and the supernatant was filtered with a 0.45 μm filter. The protein content of residual filtrate was determined with Bio-Rad RC DC (Reducing agent Compatible Detergent Compatible) protein assay kit I (500-0121). Four buffers were used: 0.1 M sodium phosphate buffer, pH 7.0; 0.1 M sodium phosphate buffer plus 2% sodium dodecyl sulfate (SDS); 0.1 M sodium phosphate buffer plus 1% 2-mercaptoethanol (2ME); and 0.1 M sodium phosphate buffer with 2% SDS and 1% 2ME. Extractions in different buffers were independently performed in triplicate. Protein solubility was determined as:

$$\text{Protein solubility } (\%) = \frac{(\text{Protein in supernatant, g})}{(\text{Protein in starting material}, g)} \times 100 \quad (1)$$

The starting material was extruded FFSF or pressurized FFSF.

High Pressure Size Exclusion Chromatography

The supernatant recovered from the extraction in the selected buffers was adjusted to 2 mg/mL prior to analysis by high pressure size exclusion chromatography (HPSEC). The proteins were separated on a Bio-Rad Bio-silSEC 400-5 column (300 × 7.8 mm) with a Bio-Rad Bio-silSEC 400 guard column (80 × 7.8 mm) upstream. The mobile phase used was 0.05 M NaH_2PO_4, 0.05 M Na_2HPO_4, 0.15 M NaCl pH 6.8 in nanopure water at a flow rate of 1.0 mL/min. Injection of 50 μL of the protein solution was performed and the absorbance was followed at 280 nm. Molecular weight calibration was achieved using a set of molecular weight protein standards within the molecular weight range of 1.3 and 670 kDa obtained from Bio-Rad (151-1901). The Galaxie software (version 1.9, Varian, Inc. Walnut Creek, CA, USA) was used to analyze the data.

Data Analysis

The general linear model PROC GLM in the Statistical Analysis Software (SAS) (version 9.1, SAS Institute, Inc., Cary, NC, USA) was used to determine the least significant difference (LSD) between means at a 5% level of probability.

Results and Discussion

In this study, extrusion and pressurization were applied to full-fat soybean flakes (FFSF) prior to aqueous extraction processing, assisted or not with a protease. Analyses were performed to establish mechanisms involved during treatment and how they correlate with protein and oil extractability. Extrusion has been used for many years for the production of ready-to-eat cereals, snacks, and food additives. An extruder generally consists of a fixed metal barrel, containing one or two screws, through which the material is axially transported from the feed end to the other end (Fig. 1). Heat is generated using heaters and through friction caused by shear stress during passage of the material through the barrel. Pressure builds up throughout the barrel as food exits the extruder, and steam is flashed due to pressure differential. With HPP, pressure is applied isostatically and therefore the sample shape is usually maintained. Heat is generated during treatment due to adiabatic heating [19] and can also be added to the system with the use of external heater. To identify the nature of protein interactions, the treated FFSFs were extracted by different solubilizing buffers, and supernatants recovered from these extractions were analyzed. Due to technology constraints, FFSF was dispersed into water or selected buffer and placed into flexible pouches for HPP treatment. With extrusion, the FFSF was first extruded and then dispersed into water or a selected buffer. During the water extraction, which was performed at a flake-to-water ratio of 1:10 regardless of the pretreatment applied, the activity of Protex 7L upon the starting material was assessed by measuring the degree of hydrolysis and observing the peptide profile of the extracted protein. After the extraction into water, the aqueous phases were separated from the unextractable material, that is the insoluble fraction, by centrifugation. To evaluate the effect of processing on the soybean flake structure, the insoluble fractions were observed using light microscopy.

Light Microscopy

The microscopic analysis demonstrated that regardless of the treatment applied, all insoluble fractions contained some seed coat and associated cell layers. This observation illustrated that the single dehulling step applied during the soybean flake preparation was insufficient for total removal of the hulls (results not shown).

Extraction from FFSF

In the insoluble fraction recovered from the aqueous extraction of untreated FFSF, both intact and ruptured palisade-like cells were observed (Fig. 2a). This observation confirmed that flaking is promoting the disruption of some, but not all, of the cotyledon cells [20]. As expected both lipid and protein materials were observed in the cotyledon cells of the insoluble fraction. The lipid droplets, which appeared as gray circles, were embedded in the

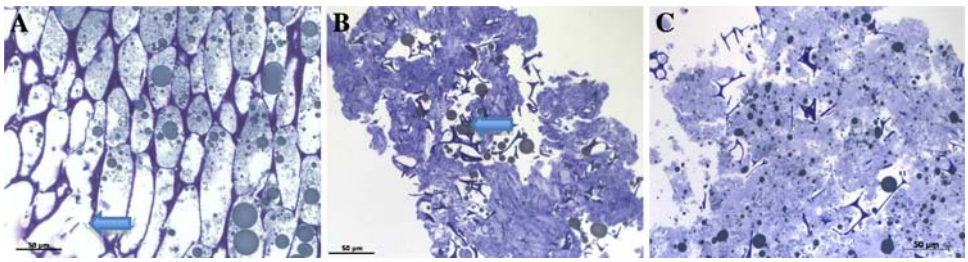

Fig. 2 Microscopic observations of insoluble fractions recovered after aqueous extraction processing of **a** full-fat soybean flakes (FFSF). *Arrow* shows empty broken cell. **b** extruded FFSF. *Arrow* shows entrapped cell wall fragment **c** 500-MPa FFSF

cytoplasmic network of unruptured cells. In unprocessed mature soybeans, lipid bodies ranged in size from 0.2 to 0.5 μm. Lipid droplets as large as 100 μm to less than 10 μm were observed, which features suggest coalescence of the lipid bodies during aqueous extraction. The lipid bodies are usually spherical in shape and considerably smaller than protein bodies, which ranged in size from 5 to 10 μm [20, 21]. The proteinaceous material was dispersed into the cytoplasm of the cells, probably due to the swelling and eventual rupture of protein bodies during aqueous extraction [20].

Extraction from Extruded FFSF

Extrusion promoted dramatic changes in the FFSF insoluble fraction, with almost no intact cells remaining (Fig. 2b). Only a few cells that were close to the remaining cell wall were intact (results not shown). The thicker membrane of these cells probably protected them from the disruption process that occurred during extrusion [22]. Many of the oil droplets released due to the loss of the cotyledon cell integrity during extrusion were entrapped in insoluble protein aggregates. Indeed proteins aggregated into a number of large discontinuous masses with many of them containing entrapped cell wall fragments and oil droplets. Therefore, the previously reported moderate increase in oil extraction yield, i.e., from 60 to 67%, obtained after extrusion of FFSF [16] was not due to a lack of cell disruption, but due to the entrapment and/or interaction between protein and oil. This observation explained the earlier results of Lamsal et al. [23], who reported an oil extraction recovery decrease during AEP of extruded flour from 75 to 54%, when barrel temperature of the extruder was increased from 100 to 120 °C. As suggested by these authors, our results showed that a higher temperature might have promoted more entrapment of the oil droplets in the insoluble protein aggregates and/or stronger bondings between oil and proteins. Similarly, the formation of large insoluble aggregates during extrusion explained the low 38% protein extractability obtained with extruded FFSF compared to 62% with the untreated FFSF [16]. In untreated FFSF, moderate protein and oil extraction yields were due to a physical barrier from the intact or not totally ruptured flaked cotyledon cells. During extrusion, both oil and proteins were released from the cotyledon cells. However, the proteins precipitated during treatment and entrapped the released oil droplets. Extrusion also drastically modified the appearance of the insoluble proteinaceous material in the insoluble fraction. The proteins were stretched and aligned in sheets, which can be ascribed to the shearing action of the rotating screws of the extruder [5, 24].

Extraction from Pressurized FFSF

After pressurization, the cellular organization of the cells in the insoluble fractions from FFSF treated at 200 and 500 MPa was similar to the one of the control. Similarly to what was observed after extrusion, the treatment at 500 MPa promoted the insolubilization of the protein. Oil droplets were trapped in the protein aggregates (Fig. 2c). Some of the insoluble protein aggregates were outside the cellular structures. It could therefore be concluded that the precipitated proteins were the proteins extracted from cells broken due to flaking. The proteins and oil from these cells first migrated into the water phase before HPP treatment. The 500-MPa treatment precipitated the proteins which entrapped some of the oil. The low flake-to-water ratio of 1:3 used to disperse the flakes before HPP treatment probably contributed to the physical interactions between protein and oil. This observation explained the decrease in protein and oil extractability observed after AEP of 500-MPa treated FFSF when compared to the untreated FFSF [16]. Contrary to what was observed in the insoluble fraction of extruded FFSF, the aggregates of the pressurized samples did not have as much cell membrane debris, which agreed with the observation of no apparent increased cellular disruption. The appearance of the proteinaceous material was similar to the control.

Extraction with Protease (EAEP)

The addition of protease Protex 7L during the aqueous extraction of untreated and 200-MPa treated FFSF did not modify the appearance of the insoluble fractions compared to the ones obtained without enzyme (results not shown). For the extruded FFSF, the aggregates were seen at a lower frequency and had a different appearance, i.e., there were

very few oil droplets entrapped in these structures and they had a lighter staining intensity, which suggested the presence of less protein. The protease was therefore efficient in breaking some of the oil/protein, protein/protein and/or protein/cell wall interactions induced by extrusion. After enzymatic treatment of the FFSF pretreated at 500 MPa, all the protein aggregates previously observed disappeared. The enzyme was therefore efficient in breaking all pressure-induced protein aggregates located outside the cell structure. Oil and protein extraction yields of ~60 and ~75%, respectively, were previously reported for EAEP of both 500-MPa FFSF and untreated FFSF [16]. This result supported that addition of Protex 7L to the 500-MPa pretreated sample solubilized precipitated proteins and released entrapped oil present in these aggregates, but did not act on the oil or proteins that were still present in the cotyledon cells.

Extent of Protein Hydrolysis During Enzyme-Assisted Aqueous Extraction of Full-Fat Soybean Flakes

For all samples, the degree of hydrolysis (DH) upon addition of Protex 7L increased sharply during the first 10 min and then slightly increased to reach a plateau (Fig. 3).

Extraction with FFSF and Pressurized FFSF

Elevated pressure, i.e., 500 MPa, induced protein reconfiguration/modification that favored proteolytic attack as illustrated by the degree of hydrolysis (DH) of 5.6 ± 0.4% obtained with 500-MPa FFSF versus 3.6 ± 0.6 for 200-MPa FFSF (Fig. 3).

The polypeptide profile of the proteins extracted with AEP of untreated FFSF obtained by SDS-PAGE displayed the traditional soy protein profile with the presence of the α', α and β subunits of β-conglycinin and the acidic and basic polypeptides of the glycinin (Fig. 4). None of the pressure levels, i.e., 200 and 500 MPa, affected the profile of the proteins recovered after AEP. This observation agreed with previous studies observing no effect of high pressure on the polypeptide profile of soy protein [26].

When the protease was added during the extraction step of untreated FFSF, the α' subunit of the β-conglycinin disappeared simultaneously with the appearance of a band at ~50 kDa along with multiple bands in the area above 20 and below 34 kDa (Fig. 4). Similar modifications of the protein profiles were observed for EAEP of 200-MPa FFSF. An increase in the pressure level to 500 MPa dramatically affected the polypeptide profile of the EAEP extracted proteins, with disappearance of almost all β-conglycinin and glycinin subunits and new bands appearing in the 20–34 kDa area. Therefore enhanced proteolysis of soy proteins occurred only after a treatment at elevated pressure, i.e., 500 MPa. Similar observations were reported during proteolysis of pressurized β-lactoglobulin [27].

Extraction with Extruded FFSF

Extrusion of FFSF promoted protein hydrolysis as illustrated by the DH value of 4.3 (Fig. 3). This observation agreed with results obtained with extruded soy concentrate and soybean meals [8, 10, 25]. Interestingly, the increased DH value for the enzyme-treated extruded FFSF was not reflected in the peptide profile of the sample. Indeed the peptide profile of the untreated FFSF and extruded FFSF recovered after EAEP were similar (Fig. 4). These results combined with our microscopic observations suggested that refolding/aggregation of the extruded proteins enhanced enzymatic hydrolysis during aqueous extraction compared to untreated FFSF, but these changes did not modify further the polypeptide profile when compared to

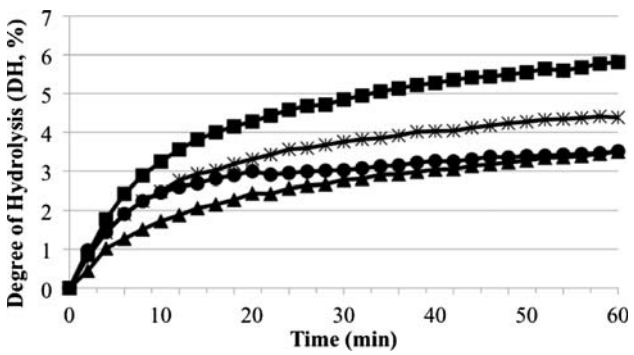

Fig. 3 Degree of hydrolysis during enzyme-assisted aqueous extraction of FFSF, extruded FFSF and pressurized FFSF. EAEP was performed with 0.5% Protex 7L at 50 °C. *filled triangles* FFSF, *filled circles* 200-MPa FFSF, *asterisks* extruded FFSF, *filled squares* 500-MPa FFSF

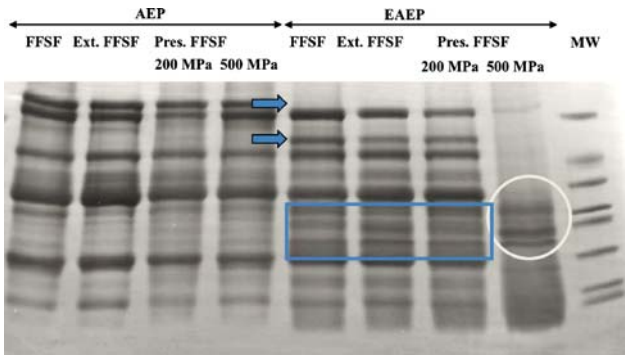

Fig. 4 SDS PAGE of proteins from aqueous extraction processing (AEP) and enzyme-assisted aqueous extraction processing (EAEP). *FFSF* full-fat soybean flakes, *Ext. FFSF* extruded FFSF, *Pres. FFSF* pressurized FFSF, *MW* Molecular weight standard; 66, 45, 36, 29, 24, 20, 14.2. 6.5 kDa

Table 1 Protein solubility of extruded and pressurized full-fat soybean flakes in different extraction buffers

Extraction buffers	Protein solubility (%)		
	Extruded FFSF	Pressurized FFSF	
		200 MPa	500 MPa
Phosphate buffer (B)	12.59 a	41.70 a	12.99 a
B + 2% SDS	41.56 b	48.20 a,b	43.52 b
B + 1% 2ME	25.89 c	45.71 a,b	34.04 c
B + 2% SDS + 1% 2ME	50.27 d	50.35 b	49.34 d
LSD	4.00	7.08	5.49

Values in a column bearing the same letters are not statistically different ($p > 0.05$)

FFSF full-fat soybean flakes, *SDS* sodium dodecyl sulfate, *2ME* 2-mercaptoethanol

the one obtained with untreated FFSF. It is more likely that the increased hydrolysis was due to the action of the protease on the new protein/protein and protein/lipid bondings.

Effect of Solubilizing Buffers on Protein Extractability and Molecular Weight Distribution of Soluble Proteins

The protein solubility of the extruded FFSF, 200-MPa FFSF, and 500-MPa FFSF was determined in the presence of agents known to disrupt noncovalent interactions, i.e., SDS, or to reduce disulfide bonds, i.e., 2-mercaptoethanol (2ME, Table 1). Solubility in phosphate buffer was used as a baseline for comparison.

Protein Extractability

Extraction with Pressurized FFSF

For the 200-MPa pressurized samples, there was a small but significant protein solubility increase when extracted with SDS + 2ME, while individual buffers did not significantly modify protein extractability. The protein solubility of the 500-MPa FFSF increased by approximately 3.3-fold with SDS, and 2.6-fold with 2ME. This result illustrated that noncovalent interactions and disulfide bonds were responsible for the low protein solubility (12.99%) of this sample. The simultaneous addition of the disulfide and noncovalent breaking agents gave the highest solubility. The protein solubility was limited to ~50% due to the physical barrier of the cotyledon cells.

Extraction with Extruded FFSF

The solubility of extruded flakes was 3.3-fold higher with SDS addition and 2.0-fold with 2ME addition. Highest solubility (50.27%) was obtained with SDS + 2ME, suggesting a synergistic effect of the two reagents, which can be explained by increased access of the 2ME once noncovalent interactions were disrupted with SDS [7].

HPSEC Profiles

The molecular weight distribution of the protein extracted from FFSF, extruded FFSF, and pressurized FFSFs in

Table 2 Effect of extraction buffers on proportion (%) of molecular weights of extracted proteins

Extraction buffers	Group[a]	FFSF	Extruded FFSF	Pressurized FFSF	
				200 MPa	500 MPa
Phosphate buffer (B)	1	28 b	10 a	32 bc	5 a
	2	14 b	7 a	14 b	24 c
	3	58 a	82 c	54 a	71 b
B + 2% SDS	1	40 bc	34 ab	46 c	47 c
	2	10 ab	12 ab	11 ab	13 b
	3	50 bc	54 c	43 ab	40 a
B + 1% 2ME[b]	1	69 bcd	74 cd	65 b	18 a
	2	31 abc	26 ab	35 c	81 d
B + 2% SDS	1	76 a	68 ab	67 b	66 b
+1% 2ME[b]	2	24 a	32 ab	33 b	34 b

Values in a row bearing the same letters are not statistically different ($p > 0.05$)

B phosphate buffer, *SDS* sodium dodecyl sulfate, *2ME* 2-mercaptoethanol, *FFSF* full-fat soybean flakes

[a] Data came from HPSEC chromatograms. Groups 1, 2 and 3 include molecular weights between 670 and 158 kDa, between 158 and 17 kDa, and lower than 17 kDa, respectively

[b] The 2ME elutes into the group 3, therefore, this group was not included in the calculation when 2ME was added into the 0.1 M phosphate buffer. Statistical analysis was performed independently on results obtained with 0.1 M phosphate buffer without 2% 2ME, and results with 0.1 M phosphate buffer including 2% 2ME

different extracting buffers was determined after dilution and elution in 0.1 M sodium phosphate buffer (Table 2).

Extraction with FFSF and Pressurized FFSF

The HPSEC chromatogram was divided into three groups corresponding to molecular weights between 670 and 158 kDa, between 158 and 17 kDa, and lower than 17 kDa, corresponding to groups 1, 2 and 3, respectively (Table 2). Group 1 of FFSF displayed one major peak that was identified as glycinin. Glycinin has a molecular weight of approximately 360 kDa, which agrees with our result. β-Conglycinin has a molecular weight of 150–200 kDa, and no major peak was observed in this area. This observation suggested that β-conglycinin might be involved in soluble aggregates of much larger molecular weight. Subunits of β-conglycinin were indeed observed in the SDS PAGE. In phosphate buffer, the proportion of these three groups for FFSF was 28, 14 and 54.

The treatment at 200 MPa of the FFSF had no significant impact on the profile and proportion of the HPSEC chromatogram, which agreed with results reported on soy protein isolate treated at the same pressure level [12]. A 500-MPa treatment, however, significantly affected the HPSEC of extracted proteins. A decrease of high molecular weight polypeptides (group 1) was observed along with an increase of the proportion of low molecular weight peptides (group 3). Meanwhile, treatment at 500 MPa also increased the proportion of intermediate polypeptides (group 2). At pH 7.6 glycinin is an hexameric complex formed of acidic (A) and basic (B) subunits linked by a single disulfide bridge (A_n-S–S-B_n), while β-conglycinin is a trimer resulting from seven possible combinations between α', α and β subunits. The acidic, basic and α', α and β subunits have a molecular weight of 38, 20, 58–83, 58–77 and 42–53 kDa, respectively. Upon heating, the subunits of the proteins are dissociated and interactions between dissociated subunits of 7S and 11S, mainly β-7S and B-11S, led to formation of a soluble complex. An increase in the proportion of the group 3 of the 500-MPa pretreated samples suggested dissociation of the proteins, which would agree with the results of Wang et al. [28]. However, the molecular weights of group 3 were lower than 17 kDa, and therefore smaller than the individual subunits of glycinin and β-conglycinin.

After adding SDS or 2ME the profile of the extract from 200-MPa pretreated FFSF remained similar to the one obtained for the untreated FFSF in the same buffers. For the 500-MPa pretreated FFSF, the profile of the proteins extracted with SDS was similar to the untreated FFSF obtained with the same buffer. This similarity between FFSF and 500-MPa pretreated FFSF was not observed after 2ME addition. In this case, the largest proportion was obtained for group 1 for the FFSF and for group 2 for the 500-MPa pretreated FFSF. This result suggested the solubilization of β-conglycinin after adding 2ME, and therefore that β-conglycinin aggregates induced by treatment at 500 MPa involved disulfide bonds, but further investigations are required to verify this hypothesis.

Extraction with Extruded FFSF

As observed for the 500-MPa pretreatment, when extruded sample was extracted in phosphate buffer, the proportion of group 1 and 3 decreased and increased, respectively, compared to the untreated FFSF (Table 2). There is an important body of literature reporting dissociation/aggregation of soy proteins during extrusion leading to decreased solubility [6–10]. This decrease in solubility could be due to the formation of high-molecular weight aggregates [28]. SDS profile of the extracted proteins was not changed by extrusion so it is more likely that the increase of the proportion of small molecular weight molecules was a consequence of the involvement of the molecules of group 1 in large aggregates that were not detected in the experimental conditions used. After addition of SDS, 2ME and SDS + 2ME, the molecular weight distribution of the proteins extracted from extruded FFSF was not significantly different from the one of the untreated FFSF. This observation was significantly different from what we observed with 500 MPa, illustrating the difference in protein interactions promoted by each treatment.

Conclusions

Our study provided evidence that extrusion of FFSF promoted cellular disruption which moderately increased oil recovery during aqueous extraction of extruded full-fat soybean flakes because oil released from the cotyledon cells was simultaneously entrapped in protein aggregates. A similar phenomenon (entrapment of oil in protein aggregates) occurred during treatment of FFSF at 500 MPa, but to a lesser extent as cotyledon cells were not disrupted by the treatment. The protease added during EAEP of the extruded and 500-MPa treated FFSF released the oil from these aggregates, increasing considerably the oil extraction yield of the extruded material [16]. This study established that high-pressure processing at 200 and 500 MPa was not efficient as a pretreatment of full-fat soybean flake to improve oil and protein extraction yields during EAEP. The characterization of the structural changes and protein modifications occurring during extrusion will contribute to the development of EAEP of extruded FFSF, which has become an attractive green alternative to hexane extraction of soybean oil.

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