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ORIGINAL PAPER

Quantity and Quality of Free Oil Recovered from Enzymatically Disrupted Soybean Oleosomes

Lili T. Towa · Virginie N. Kapchie · Guang Wang · Catherine Hauck · Tong Wang · Patricia A. Murphy

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Abstract The operational variables impacting the quantity and quality of free oil recovered from isolated soybean oleosomes by enzymatic extraction were evaluated to optimize this process. Those variables were: protease concentration (0–2.5%), protease hydrolysis time (3 vs. 18 h), and slurry destabilization time (30 min vs. 3 h). Analysis of interactions between these variables and the yield of free oil revealed that the protease concentration was the most significant variable. The quantity of free oil extracted by using 3 h of oleosomes hydrolysis and 30 min of slurry destabilization was not significantly different from that using 18 h of oleosomes hydrolysis and 3 h of slurry destabilization. The optimum conditions, 0.5% Protex 6L, 3 h of hydrolysis, and 30 min of destabilization, resulted in 90% free oil. Oils extracted by the aqueous process had a fatty acid composition similar to conventional hexane-based process with oxidative stability indices ranging from 9 to 12 h. Enzyme assisted aqueous extraction resulted in a high quality oil which has 88% less free fatty acids than hexane-extracted oil. The optimal conditions generated 85.5% soybean storage proteins in skim with peptides smaller than 6.5 kDa and the degree of hydrolysis of 19.5%. The present study demonstrates that oil can be extracted from soybeans efficiently without hexane or other petroleum solvents.

Keywords Soybean oleosomes · Soy oil · Aqueous-extraction · Oil quality

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Introduction

Solvent extraction is the primary means of extracting oil from oil seeds in the food industry. Safety considerations and clean air regulations regarding the use of organic solvents prompted development in the past of aqueous extraction processes as environmentally friendly and safer alternatives. The aqueous extraction process involved a simultaneous extraction of free oil and protein from oilseeds in an aqueous medium [1, 2]. Although the concept appeared potentially attractive, the yield of free oil extracted was lower than that from the conventional hexane-based process [1–3]. To overcome the low oil extraction efficiency, several research groups explored the use of hydrolytic enzymes in the process which help increase the yield of free oil [4–9]. However, particularly for the soybean, the release of oil into the aqueous medium results in a stable oil-in-water emulsion with soy protein and phospholipids acting as surfactants [10]. Lamsal and Johnson [11], Chabrand et al. [12], and Wu et al. [13] used different techniques including freeze–thaw, heating and enzymatic processes to destabilize the emulsions, but with difficulties. An alternate pathway to oil isolation by aqueous means was developed by Towa et al. [14], namely enzyme assisted-aqueous extraction (EAAE) of oil from isolated soybean oleosomes. Oleosomes or oil bodies are discrete subcellular organelles mainly found in oilseeds. They consist of a triglyceride core surrounded by a phospholipid monolayer in which the oleosins are embedded. The oleosins are unique proteins that stabilize the oleosome structure and prevent oil coalescence in the cell cytoplasm [15, 16]. Our group demonstrated that large amounts of oleosomes can be successfully isolated from 75 kg of full fat soybean flour on a pilot plant-scale [17]. These isolated oleosomes were not a true emulsion but instead they

appeared to be isolated subcellular organelles, lipid reservoirs surrounded by an intact membrane. For these reasons, we hypothesized that free oil could be released from those organelles by disrupting the membrane with proteases, phospholipases, and a combination of thermal (freeze–thaw, heating) and mechanical destabilization of the slurry and cream [14]. The most efficient strategy was shown to be hydrolyzing the oleosomes' membranes with 2.5 or 5% Protex 6L for 18 h, destabilizing the slurry by heating at 96 °C for 3 h and destabilizing the resulting cream by the butter-churning process resulting in a yield of 84% of free oil. While promising, further optimization of this developing technology is needed to address process related issues such as time, enzyme concentration, and the quality of the final product.

The objective of this study is to optimize our EAAE of oil from isolated oleosomes by evaluating the effects of the operating conditions, % proteases, oleosome membrane hydrolysis time, and slurry destabilization time on the oil recoveries and the qualities of the recovered oil and protein.

Materials and Methods

Materials

Oleosomes were isolated in the pilot plant of the Center for Crops Utilization Research, Iowa State University from 75 kg of full-fat soybean flour. The flour was obtained in 2009 from Natural Products Inc (Grinnell IA, USA) and was stored at 4 °C for 1 week prior to use. Briefly, the isolation process involved the use of cell wall hydrolyzing enzymes, mechanical disruption of cell walls through the Stephan Microcut® mill (Stephan Machinery Corp., Columbus, OH model MC-10), and flotation centrifugation through the Three Phase Centrifuge (Centrysis, Model 10/4, Kenosha WI, USA) that was reported elsewhere [17].

The enzyme used to disrupt oleosome membranes was Protex 6L® [EC 3.4.21.62, alkaline serine endopeptidase, 580,000 DU (Digesting Units)/g, optimal pH 9.5, optimal temperature 60 °C] as recommended by Genencor Inc. (Genencor, a Danisco company Rochester, NY, USA). Other enzymes such as bromelain, trypsin, and phospholipase A2 (Multifect L1 10L) from Bio-Cat Inc. (Virginia, USA), Sigma (St Louis, USA), and Genencor, respectively, were evaluated. However, they were not as efficient as Protex 6L.

The commercial soybean oil was purchased locally (Oil Hy-Vee 100% Vegetable Oil®). Hexane-extracted soybean oil (crude oil) and hexane-extracted freeze-dried oleosome oil were obtained by the procedure of Megahad & Kinamy [18]. Prior to extraction, the freeze-dried oleosomes were ground with a coffee mill for 1 min at maximum speed to pass through a No. 60 sieve (Hogentogler & Co., Inc., MD, USA). Then, the oils from soybean and freeze-dried oleosome flours were extracted with the HPLC grade hexane (ratio 1/6; dw/v) at 250 rpm for 2 h. The suspensions were filtered through a filter paper Whatman No. 4 (Fisher Scientific, Pittsburgh, PA, USA) and the residue re-extracted three times as described above. The hexane extracts were combined and dried in a rotary vacuum evaporator at room temperature. The oils were dried under a stream of nitrogen, and stored at 4 °C until analysis.

Analytical grade reagents used were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma (St Louis, USA).

Aqueous Extraction of Oil from Isolated Oleosomes

The oil extraction from isolated oleosomes was carried out according to the procedure by Towa et al. [14] with several modifications. The major differences between Towa et al. [14] and the procedure reported here were: a reduction in the ratio of oleosomes to water from 1/6 to 1/3 (dw/v); a reduction of the stirring rate of the slurry from 40 to 30 rpm; and elimination of the butter-churning process. These parameters were adjusted to improve the free oil yield and to reduce the process time. In fact, preliminary studies of the aqueous extraction of oil from isolated oleosomes demonstrated that the ratio oleosomes to water as well as the stirring speed influence significantly the yield of free oil (data not shown).

This current oil extraction procedure involved hydrolyzing the isolated oleosome protein membranes in a 20-L reaction vessel (Model CG-9253-10, Chemglass Inc., Vineland, NJ, USA) (Fig. 1). A mass of 1.4 kg (wet w) of isolated oleosomes (48–50% dry matter) was dispersed in distilled water (ratio 1/3; dw/v) at 30 rpm, 60 °C. The pH of the dispersion was adjusted to 9 with 2 N NaOH. When optimal conditions for Protex 6L activity were reached at 60 °C and pH 9, 0–2.5% (v/dw) enzyme was added and the mixture incubated for 3 or 18 h with constant stirring at 30 rpm. The pH of the slurry was maintained at 9 with the 2 N NaOH by the pH-stat (702 SM Titrino, Metrohm Ltd., Herisau, Switzerland). After 3 or 18 h of hydrolysis, the slurry was destabilized by heating at 96 °C for 30 min or 3 h. Then, the preparation was centrifuged using a fixed angle rotor (Sorvall RC 5B 120 Plus, L.P., Newtown, USA) at 10,000×*g* for 15 min at 25 °C to separate free oil, cream and skim. The cream represents the small middle phase residing between the free oil and skim fractions. The skim represents the aqueous fraction that contains emulsified oil which resides below the cream phase. The resulting cream was re-centrifuged as described above to separate the residual free oil trapped in the cream. The separation of

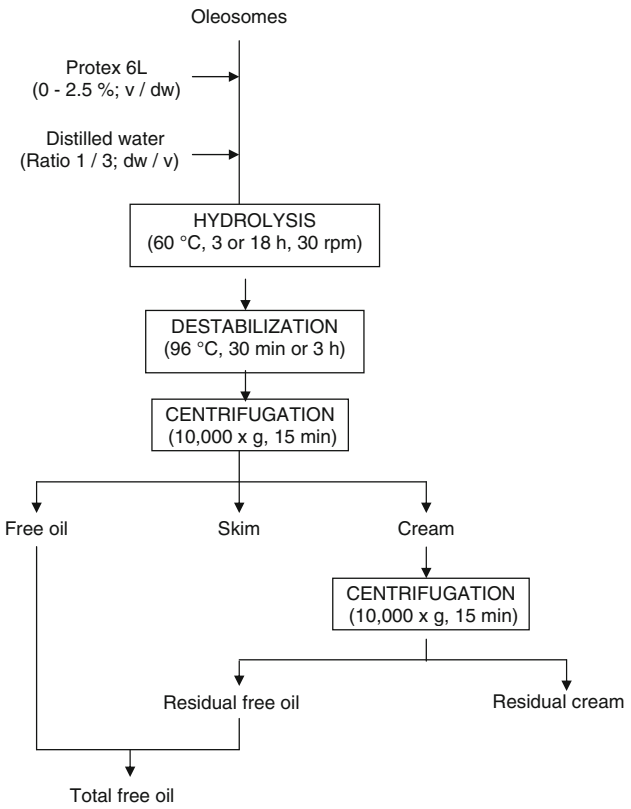

Fig. 1 Flow diagram of the enzyme assisted-aqueous extraction procedure for oil from isolated oleosomes

free oil, cream and skim was done using a glass separatory funnel at 4 °C for 24 h as described by Lamsal and Johnson [11]. The total free oil was the combination of free oil released after destabilization of the slurry, and re-centrifugation of the cream.

Analyses

Degree of Hydrolysis

The degree of protein hydrolysis (DH) in the oleosome suspension (oleosomes, protease, water) incubated at 60 °C for 3 or 18 h, pH 9, was determined by the pH-stat method [19] using the milliequivalents of the base consumed. A similar suspension without protease was used as the control. The procedure consisted of determining the %DH of protein on the basis of the number of free titratable carboxylic acid groups produced by the hydrolysis of peptides bonds. The degree of protein hydrolysis was calculated using the equation: $DH = [(V_{NaOH} \times N_{NaOH})/(\alpha \times MP \times h_{tot})] \times 100\%$, where α is the degree of dissociation of α-amino groups bonds, MP is the mass of protein (g), and h_{tot} is the number of peptides bonds in the substrate (mequiv/g protein). The NaOH concentration was 2 N, and the α value was 0.98 for the hydrolysis temperature of 60 °C and pH 9.0. The h_{tot} value for soy proteins was 7.8 [19, 20]. Protein content was evaluated with the Dumas method using a rapid N III Nitrogen Analyzer (Elementar Americas, Inc. Mt. Laurel, NJ, USA), and was calculated as total nitrogen × 6.25 [21]. Protein recovery was expressed as the percentage of protein in each fraction relative to the initial amount of protein in isolated oleosomes.

Oil content and oil recovery

The oil content of different aqueous fractions was determined by the Mojonnier method [22] to monitor the effects of the operating conditions on the oil recovery from total oil in isolated oleosomes. Oil recovery was expressed as the percentage of oil in each fraction relative to the initial amount of oil in isolated oleosomes.

Quality of Oil

The fatty acid composition, free fatty acid content, phosphorus content, and oxidative stability index of the total free oil were evaluated to determine the effects of operating conditions on the quality of oil. Commercial soybean oil, hexane-extracted soybean oil and hexane-extracted freeze-dried oleosome oil were used as the controls.

The fatty acid composition of oil was determined by the method of Eller and King [23] using a capillary gas chromatograph (GC) (Hewlett-Packard model 5890 series II gas chromatograph with a flame ionizer detector) after preparation of fatty acid methyl esters [24]. An oil sample of 0.2 g was dissolved in 4 mL of sodium methoxide (1 M in methanol). The reaction was conducted at 45 °C for 1 h in a force-convection oven and was stopped by adding two drops of distilled water. Fatty acid methyl esters were extracted three times using hexane and applied to GC for analysis. The GC conditions were as follows: injection temperature, 230 °C; detector temperature, 230 °C; oven temperature programmed from 110 to 220 °C with a heating rate of 10 °C/min. The column used was a Supelco SP-2330 (Bellefonte, PA, USA) capillary column, 15 m (length) × 0.25 mm (i.d.) × 0.2 μm (film thickness). The oxidative stability of the free oil was evaluated using the Oxidative Stability Instrument (Omnion Inc., Maple and Plain Street, Rockland, MA, USA) using 5-g oil samples at 100 °C, and the air flow of 8.13–8.19 L/h, according to American Oil Chemists' Society (AOCS) Official Method Cd 12b-92 [25]. The official AOCS [25] method Ca 12–55 was used to determine the phosphorus content in the free oil samples. Free fatty acids (FFA) were isolated from free oil by using thin layer chromatography (TLC) plates (Silica Gel G 500, Analtech Inc., Newark, DE) and hexane/ethyl ether/formic acid (80/20/2; v/v/v) as the mobile phase [26]. The free fatty acid band was detected after spraying the

plates with 2′,7′-dichlorofluorescein by viewing under UV light. Then, the free fatty acid band was scraped off the plate. Free fatty acids were converted to methyl esters with 3% sulfuric acid in methanol (v/v) at 65 °C for 3 h. The composition of free fatty acids was obtained using GC as described above. Methyl heptadecanoate was used as an internal standard for FAME and FFA quantification.

Polypeptide Profiles

Urea-SDS–PAGE was performed to determine the effects of the operating conditions on soy protein polypeptides profiles. Soy flour, oleosomes and aqueous samples from the EAAE process were prepared for the urea SDS-PAGE according to Beisson et al. [16]. First, 70 μg low molecular weight markers protein (6.5–66 kDa) was loaded as a standard, and the same quantity of protein from soybean flour, oleosomes or aqueous samples from the aqueous process was loaded in separate wells. Electrophoresis was run as suggested by Lamsal et al. [11] with a SDS-tris–glycine buffer system, 4% stacking gel and 13% resolving gel (Biorad Mini Protean II Gel). Gels were scanned on an Amersham Pharmacia Biotech Image scanner with a transparency module and analyzed with Kodak Molecular Imaging (MI) Software version 4.

Experimental Design and Statistical Analysis

To optimize the variables examined in our aqueous extraction process, a response surface design of 15 experiments was conducted in two steps (Table 1). The first step helped to evaluate interactions between operating conditions and free oil released from oleosomes using the half normal plot methodology (SAS System, version 9.2, SAS Institute, Inc., Cary, NC, USA). The second step allowed prediction of the model for free oil released from oleosomes as a function of the operating conditions, and to identify the optimum by one-way ANOVA (SAS System, version 9.2, SAS Institute, Inc., Cary, NC, USA) (Table 1). The operating conditions were: % enzymes (0–2.5% Protex 6L), hydrolysis times of oleosome membranes (3 vs. 18 h), and destabilization times of the slurry (30 min vs. 3 h) (Table 1). All experiments were performed in duplicate. The analyses on the resulting fractions were performed in triplicate. General Linear Model ANOVA, Least Significant Difference and Duncan tests in SAS System were used to compare the treatment means at $p < 0.05$.

Results

The isolated oleosomes used in this present study were intact 0.2–0.5 μm organelles consisting of a lipid core bounded by a fine membrane. They were composed of 48–50% dry matter, 50.1% oil (d.b.), 21.1% protein (d.b.), 5.5% ash (d.b.), and 22.8% carbohydrate (d.b.), by difference. Protex 6L at 0.25% resulted in lower DH than at 2.5% at both incubation times as shown in Fig. 2a. A 10-fold increase in the protease only led to a doubling of DH. These results suggested that both protease concentration and incubation time affected the oleosome disruption. Having established the effect of enzymes on DH (Fig. 2a), further evaluation, as shown in Fig. 2b, indicated the maximum protease dose to be 0.5% for a DH of 19.5%.

Protex 6L at 0.25% resulted in a lower % free oil than at 2.5% as shown in Fig. 3a. A 10-fold increase in the protease led to a 20% increase in free oil. These results suggested that enzyme concentration, hydrolysis times and destabilization times could affect the % free oil. Analysis of interactions between theses variables and the resulting free oil revealed the protease concentration as the most significant parameter. The total free oil obtained with 3 h of oleosomes hydrolysis and 30 min of slurry destabilization was not significantly different from that with 18 h of oleosomes hydrolysis and 3 h of slurry destabilization. The effects of operating variables as established in Fig. 3a, led to further evaluation, as shown in Fig. 3b. The maximum free oil of 90% was recovered with a protease dose of 0.5% (Table 2). Only 10% of free oil was extracted from oleosomes disrupted without protease. Proteases are necessary to enhance the extractability of oil from oleosomes [4, 9, 14].

Table 1 Response surface evaluation of the operating conditions for extracting oil from isolated oleosomes

Treatment combinations		
Enzymes (%)	Hydrolysis time (h)	Destabilization time (h)
Step 1		
0.25	3	0.5
0.25	3	3
0.25	18	0.5
0.25	18	3
2.5	3	0.5
2.5	3	3
2.5	18	0.5
2.5	18	3
Step 2		
0	18	3
0.25	18	3
0.5	18	3
1	18	3
1.5	18	3
2	18	3
2.5	18	3

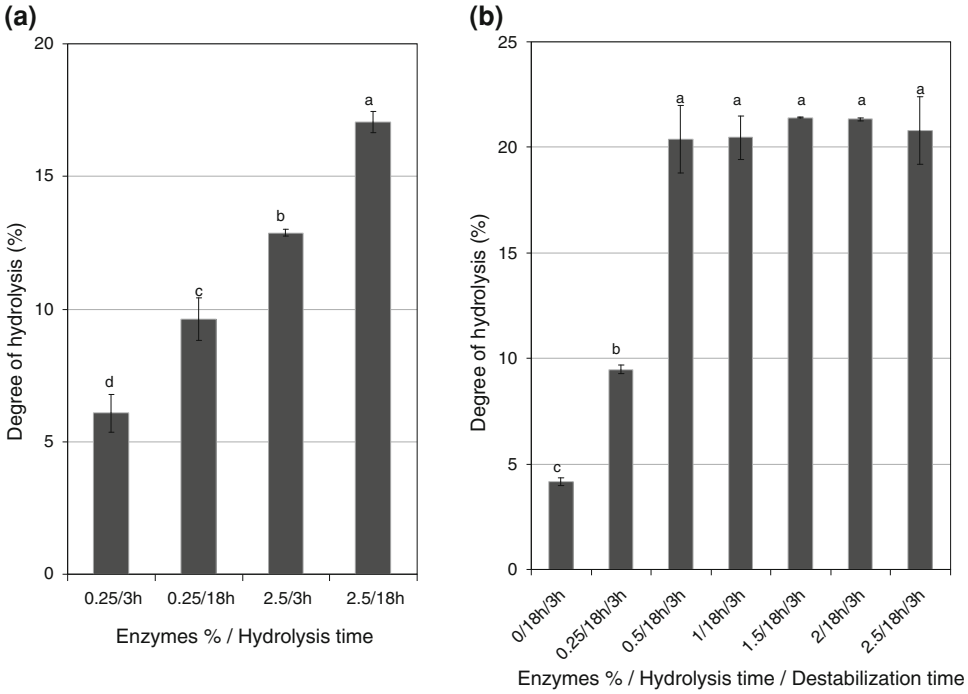

Fig. 2 Effects of % Protex 6L (**a, b**) and its reaction time (**a**) on the degree of hydrolysis of oleosome protein membranes. 0.25/3 h, 2.5/3 h: oleosomes hydrolyzed with 0.25 and 2.5% Protex 6L for 3 h, respectively; 0.25/18 h, 3 h/18 h: oleosomes hydrolyzed with 0.25 and 2.5% Protex 6L for 18 h, respectively; 0/18 h, 0.5/18 h, 1/18 h, 1.5/18 h, 2/18 h: oleosomes hydrolyzed with 0, 0.5, 1, 1.5 and 2% Protex 6L for 18 h, respectively. [a–d]Means ± SD for a given compartment sharing the same lettered superscript are not significantly different at $p < 0.05$

The optimization of the Towa [14] process improved the free oil yield from 84% using 2.5 and 5% Protex 6L to 90% using 0.5% Protex 6L. This improvement is probably for two reasons: the reduction of the ratio of oleosomes to water from 1/6 to 1/3 (dw/v) which limits emulsification; and the reduction of the stirring rate of the slurry from 40 to 30 rpm to further reduce emulsification and the decrease in free oil recovery. Our observations are in accordance with Sharma et al. [7] who demonstrated that increasing the shaking speed during the aqueous extraction of oil from peanuts lead to a decrease of free oil recovery because of the emulsification. Jiang et al. [27] showed that the ratio of material to water influenced the yield of free oil released during the aqueous enzymatic extraction of peanut oil. Although our study defined the optimal conditions to extract 90% of oil from the oleosomes (Fig. 1), the quality of the resulting oil required evaluation prior to demonstrating this process is an efficient alternative to the conventional hexane extraction.

The oil quality depends on the technology used to extract and refine it as well as the quality of the raw material [28–31]. We evaluated the effects of operating conditions (Fig. 1) on the fatty acid composition, the free fatty acid content, and the oxidative stability index of the resulting free oils. Commercial soybean oil, hexane-extracted soybean oil (crude soybean oil) and hexane-extracted freeze-dried oleosome oil were used as the controls. There was some difference between the fatty acid composition of the commercial soybean oil and that of the crude soybean oil or the hexane-extracted freeze-dried oleosome oil ($p < 0.05$) (Table 3). This may be due to the difference of the starting material used for commercial soybean oil [28–31]. The fatty acid composition of crude soybean oil was not significantly different than that of EAAE oil, produced from same starting materials, regardless of the operating conditions ($p < 0.05$). These results suggested that there was no interaction among the % Protex 6L, hydrolysis time of oleosomes, destabilization time of the slurry for the fatty acid composition of the resulting oils.

The free fatty acid content of soybean oil represents the products of enzymatic, thermal, or microbial hydrolysis of the lipids [32]. Figure 4 presents the free fatty acid contents of oils extracted by our aqueous process. The hexane-extracted freeze-dried oleosome oil had a higher % free fatty acid (1.18%) than crude soybean oil (0.22%), or aqueous extracted oils from oleosomes (0.11–0.18%) suggesting lipase activity taking place during oleosome fractionation from soybean flour. Iwanaga et al. [33] reported that soybeans contain lipases that could hydrolyze lipids to produce free fatty acids that migrate to the interface between lipids and the oleosome membranes. The commercial soybean oil had the lowest free fatty acid content (0.03%) compared to the crude soybean oil (0.22%), the hexane-extracted freeze-dried oleosome oil (1.18%), or aqueous extracted oils from oleosomes (0.11–0.18%). This low free fatty acid content of the commercial soybean oil shows the effectiveness of the degumming/neutralization steps of the commercial refining in decreasing the % free fatty acid of the crude oil, compared to our aqueous

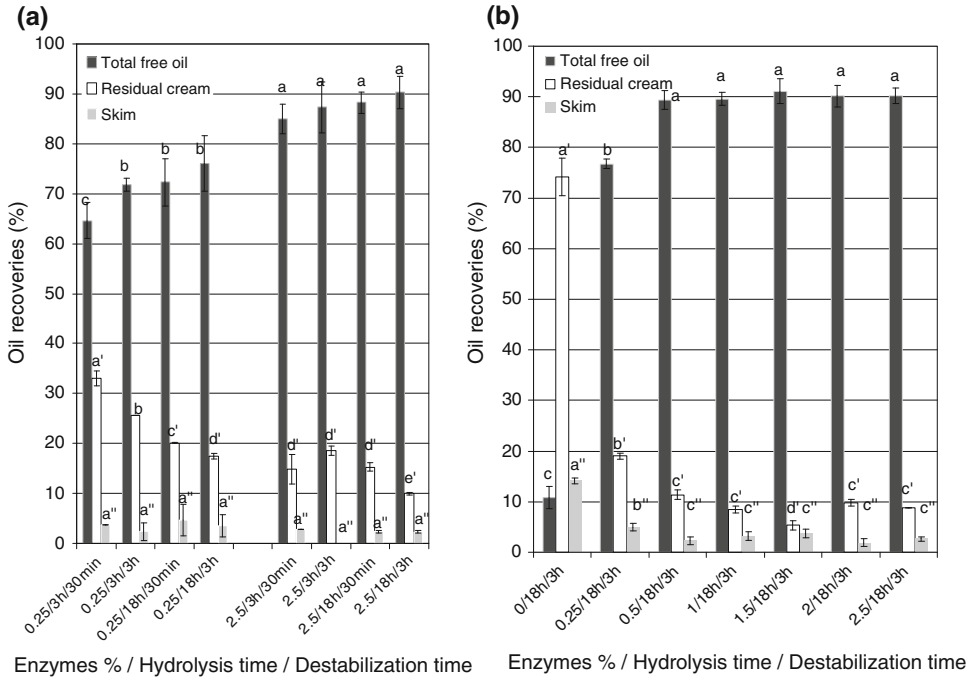

Fig. 3 Effects of % Protex 6L (**a, b**), its reaction time (**a**), and destabilization time (**a**) of the slurry on the oil distribution in different fractions. 0.25/3 h/30 min, 2.5/3 h/30 min: oleosomes hydrolyzed with 0.25 and 2.5% Protex 6L for 3 h, respectively, and destabilized for 30 min; 0.25/3 h/3 h, 2.5/3 h/3 h: oleosomes hydrolyzed with 0.25 and 2.5% for 3 h, respectively, and destabilized for 3 h; 0.25/18 h/30 min, 2.5/18 h/30 min: oleosomes hydrolyzed with 0.25 and 2.5% Protex 6L for 18 h, respectively, and destabilized for 30 min; 0.25/18 h/3 h, 2.5/18 h/3 h: oleosomes hydrolyzed with 0.25 and 2.5% Protex 6L for 18 h, respectively, and destabilized for 3 h; 0/18 h/3 h, 0.5/18 h/3 h, 1/18 h/3 h, 1.5/18 h/3 h, 2/18 h/3 h: oleosomes hydrolyzed with 0, 0.5, 1, 1.5, and 2% Protex 6L for 18 h, respectively, and destabilized for 3 h. $^{a–c,\ a'–e'\ \text{or}\ a''–c''}$Means ± SD for a given compartment sharing the same lettered superscript are not significantly different at $p < 0.05$

Table 2 Response surface prediction model for effects of % enzymes on the yield of free oil

Source	DF[a]	Squares	Mean square	*F* value[b]	Probability $p > F$
F test for one way ANOVA					
Model	6	5512.23	918.70	8908.07	<0.0001
Error	3	0.30	0.10		
Corrected total	9	5512.54			
Prediction confidence intervals at each level of enzyme percentage					
Enzymes (%)	y LSMEAN	95% confidence limits			
0	10.83	9.81	11.85		
0.25	76.43	75.70	77.15		
0.5	89.37	88.35	90.39		
1	89.58	88.86	90.30		
1.5	91.07	90.05	92.10		
2	90.17	89.14	91.19		
2.5	90.22	89.50	90.94		

[a] Degrees of freedom

[b] Significant at $p < 0.0001$

process. In fact, Jung et al. [34] reported that there was a significant decrease in % free fatty acid in crude oil after the degumming/neutralization. The refining process allows the elimination of undesirable substances such as phospholipids, pigments, and free fatty acids which may lead to an inferior quality of the final product. Regardless of the

Table 3 Effects of % Protex 6L, its reaction time, and destabilization time of the slurry on the fatty acid composition of free oil extracted from isolated oleosomes

	Fatty acid composition (%)					
	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3	Ratio unsat/sat
Controls						
Commercial soybean oil	11.44 ± 0.56^{a}	4.09 ± 0.24^{a}	21.32 ± 0.27^{b}	54.66 ± 0.88^{a}	8.13 ± 0.49^{a}	5.40 ± 0.09^{b}
Crude soybean oil	11.00 ± 1.22^{a}	3.57 ± 0.20^{b}	25.44 ± 0.52^{a}	53.10 ± 0.40^{b}	6.60 ± 0.33^{b}	5.83 ± 0.43^{ab}
Hexane-extracted freeze-dried oleosome oil	11.27 ± 0.52^{a}	3.75 ± 0.10^{ab}	26.06 ± 1.28^{a}	52.881 ± 0.5^{b}	6.51 ± 0.15^{b}	5.70 ± 0.21^{ab}
Step 1						
0.25%/3 h/30 min	11.47 ± 0.34^{a}	3.59 ± 0.06^{ab}	25.45 ± 0.09^{a}	53.23 ± 0.16^{b}	6.23 ± 0.02^{b}	5.60 ± 0.08^{ab}
0.25%/3 h/3 h	10.49 ± 0.62^{a}	3.80 ± 0.09^{ab}	25.51 ± 0.13^{a}	53.05 ± 0.01^{b}	6.52 ± 0.39^{b}	5.92 ± 0.30^{ab}
0.25%/18 h/30 min	11.91 ± 1.13^{a}	3.40 ± 0.23^{b}	24.67 ± 0.66^{a}	52.35 ± 0.71^{b}	6.16 ± 0.20^{b}	5.48 ± 0.40^{ab}
0.25%/18 h/3 h	10.97 ± 0.56^{a}	3.67 ± 0.08^{ab}	25.55 ± 0.18^{a}	53.51 ± 0.35^{b}	6.27 ± 0.05^{b}	5.84 ± 0.18^{ab}
2.5%/3 h/30 min	11.68 ± 0.44^{a}	3.60 ± 0.05^{ab}	25.11 ± 0.20^{a}	52.60 ± 0.36^{b}	6.16 ± 0.09^{ab}	5.48 ± 0.13^{ab}
2.5%/3 h/3 h	11.29 ± 0.50^{a}	3.72 ± 0.15^{ab}	25.28 ± 0.41^{a}	52.47 ± 0.25^{b}	6.43 ± 0.16^{b}	5.62 ± 0.19^{ab}
2.5%/18 h/30 min	10.81 ± 1.29^{a}	3.77 ± 0.32^{ab}	25.44 ± 0.60^{a}	52.65 ± 0.03^{b}	6.63 ± 0.02^{b}	5.82 ± 0.43^{ab}
2.5%/18 h/3 h	10.27 ± 0.38^{a}	3.76 ± 0.07^{ab}	25.46 ± 0.23^{a}	52.82 ± 0.26^{b}	6.72 ± 0.04^{b}	6.05 ± 0.17^{b}
Step 2						
0–2%/18 h/3 h	9.75 ± 0.10^{ab}	4.31 ± 0.27^{a}	26.08 ± 0.37^{a}	53.65 ± 0.42^{b}	6.69 ± 0.28^{b}	6.14 ± 0.04^{ab}

0.25%/3 h/30 min, 0.25%/3 h/3 h, 0.25%/18 h/30 min, 0.25%/18 h/3 h, 2.5%/3 h/30 min, 2.5%/3 h/3 h, 2.5%/18 h/30 min, 2.5%/18 h/3 h, 0–2%/18 h/3 h: refer to the Fig. 3 legend. ^{a,b}Means ± SD for a given oil and components sharing the same lettered superscript within the same column are not significantly different at $p < 0.05$

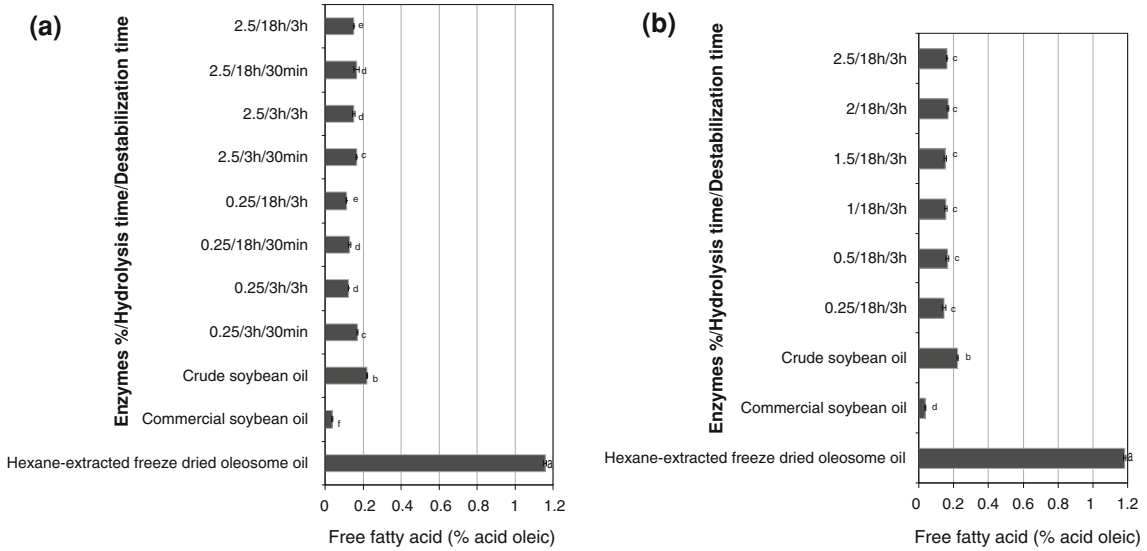

Fig. 4 Effects of % Protex 6L (**a, b**), its reaction time (**a**), and destabilization time (**a**) of the slurry on the % free fatty acid of the free oil extracted from isolated oleosomes. 0.25/3 h/30 min, 2.5/3 h/30 min, 0.25/3 h/3 h, 2.5/3 h/3 h, 0.25/18 h/30 min, 2.5/18 h/30 min, 0.25/18 h/3 h, 2.5/18 h/3 h, 0/18 h/3 h, 0.5/18 h/3 h, 1/18 h/3 h, 1.5/18 h/3 h, 2/18 h/3 h: refer to the Fig. 3 legend. $^{a–f}$Means ± SD for a given oil sharing the same lettered superscript are not significantly different at $p < 0.05$

processing conditions, oils extracted from oleosomes by the aqueous process had a higher % free fatty acid ranging from 0.11 to 0.18% than the recommended <0.05% for the fully refined soybean oil (AOCS Official Method Ca 5a 40 [25]). These oils extracted by the aqueous process contain 88% less free fatty acids than the hexane-extracted crude soybean oil. The difference can be explained by the fact that our aqueous process involved proteolytic hydrolysis and emulsion destabilization at high temperatures (65, 96 °C), during which neutralization of

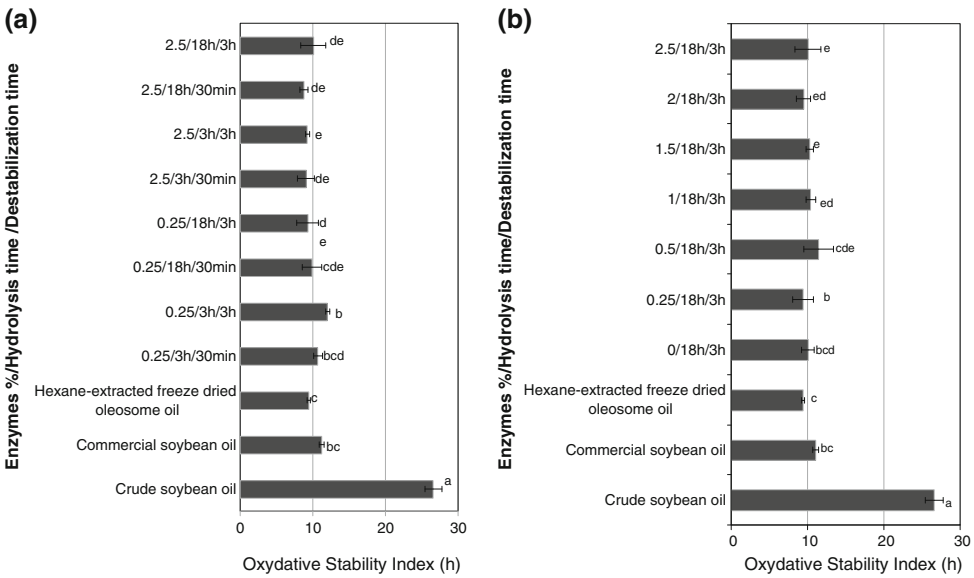

Fig. 5 Effects of % Protex 6L (**a**, **b**), its reaction time (**a**), and destabilization time (**a**) of the slurry on the oxidative stability index of the free oil extracted from isolated oleosomes. 0.25/3 h/30 min, 2.5/3 h/30 min, 0.25/3 h/3 h, 2.5/3 h/3 h, 0.25/18 h/30 min, 2.5/18 h/30 min, 0.25/18 h/3 h, 2.5/18 h/3 h, 0/18 h/3 h, 0.5/18 h/3 h, 1/18 h/3 h, 1.5/18 h/3 h, 2/18 h/3 h: refer to the Fig. 4 footnote. [a–e]Means ± SD for a given oil sharing the same letter superscript are not significantly different at $p < 0.05$

the free fatty acids occurred at the basic pH, and centrifugation could separate the water soluble soap from the free oil [11, 32, 35].

The oxidative stability index of oil is influenced by many factors including fatty acid composition, metal ions, pigments, phospholipids, % free fatty acid, mono- and diacylglycerols content, thermally oxidized compounds, and antioxidants [36]. Crude soybean oil had the highest oxidative stability index at 26.6 h compared to the aqueous extracted oils ranging from 9 to 12 h (Fig. 5). One of the reasons for this difference was probably due to the phosphorus content of crude soybean oil of 119.6 ppm which was greater than oils extracted by EAAE at 86.6 ppm. Jung et al. [34] and Bocevska et al. [37] reported that the minerals, phospholipids and antioxidants including tocopherols contents of oils extracted from corn germ and soybean, respectively, by enzymatic processes influence their oxidative stability indexes. Moreover, King et al. [38] demonstrated that the antioxidant activity of phospholipids in lipid systems is more related to their functional groups than their fatty acid composition.

There was no significant difference between the oxidative stability indexes of commercial soybean oil (11 h) and oils extracted from oleosomes by aqueous process regardless the operating conditions (9–12 h) ($p < 0.05$). These results demonstrate that the resistance to oxidation of oils extracted by our aqueous process was similar to that of commercial soybean oil. The oxidative stability indexes of oils extracted by our aqueous process meet the recommended value (>7.5 h) for refined soybean oil (AOCS Official Method Cd 12b 92 [25]). Overall, operating conditions did not affect either the fatty acid composition or the oxidative stability indexes of the free oil. The % free fatty acid decreased from 1.18% for hexane-extracted freeze-dried oleosome oil to 0.11–0.18% for oils extracted by EAAE. Our EAAE process resulted in good quality oil that was 88% lower in free fatty acids compared to hexane-extracted oil.

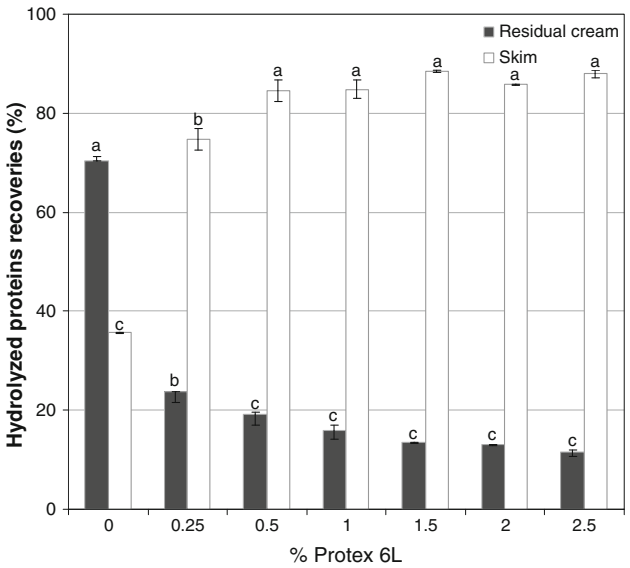

Fig. 6 Partitioning hydrolyzed proteins from isolated oleosomes between fractions of our enzyme assisted aqueous extraction process. [a–c]Means ± SD for a given fraction sharing the same letter superscript are not significantly different at $p < 0.05$

Evaluation of Oleosome Derived Protein

The distribution of hydrolyzed proteins and their composition were investigated to assess the effects of the

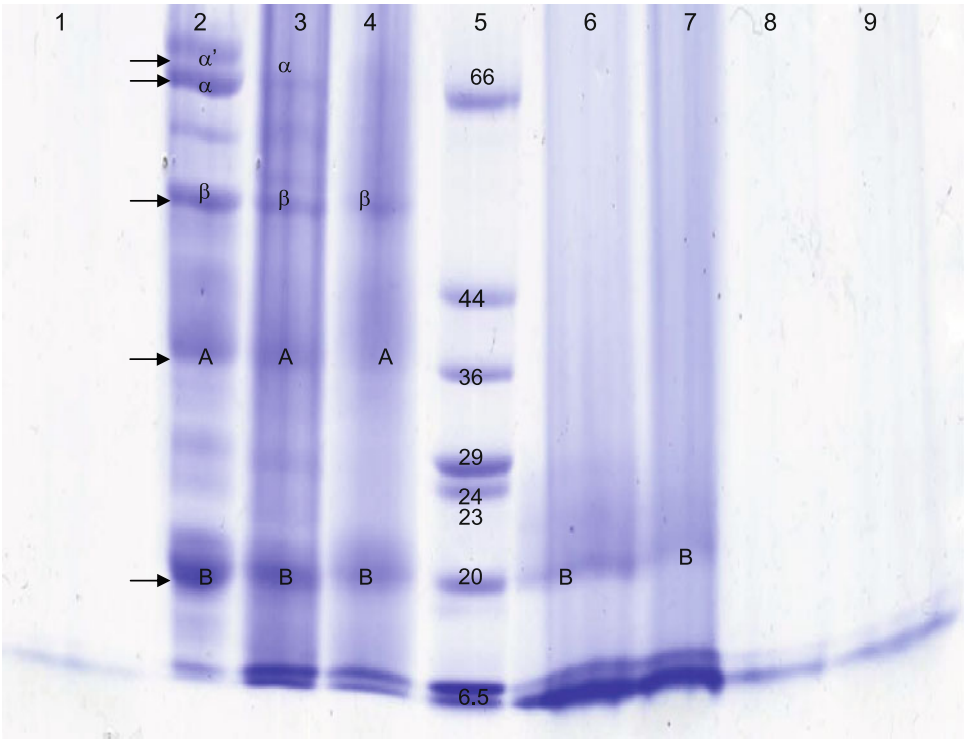

Fig. 7 Urea SDS-PAGE profiles of hydrolysates of isolated oleosomes of soybean flour. *Lane 1*, Slurry prepared with 2.5% Protex 6L for 18 h of hydrolysis and 3 h of destabilization; *Lane 2*, Soy flour; *Lane 3*, Isolated oleosomes; *Lane 4*, Slurry prepared without proteases; *Lane 5*, Low molecular markers 6.5–66 kDa; *Lane 6*, Slurry prepared with 0.25% Protex 6L for 3 h of hydrolysis and 30 min of destabilization; *Lane 7*, Slurry prepared with 0.25% Protex 6L for 18 h of hydrolysis and 3 h of destabilization; *Lane 8* , Slurry prepared with 1% Protex 6L for 18 h of hydrolysis and 3 h of destabilization; *Lane 9*, Slurry prepared with 1.5% Protex 6L for 18 h of hydrolysis and 3 h of destabilization, 80–85 μg protein/lane. α', α and β, Subunits of β-conglycinin; *A*, Acidic polypeptides of glycinin; *B*, Basic polypeptides of glycinin

processing conditions on the oleosome membranes. Except for the suspension prepared without enzymes (control), most of hydrolyzed proteins were recovered in the skim (Fig. 6). About 84–87% of hydrolyzed proteins were recovered in the skim using 0.5–2.5% protease (Fig. 6). A similar tendency was observed for the free oil recovery (Fig. 3b). These results were in accordance with those of Rosenthal et al. [39], showing that during the aqueous extraction of oil from soybean flour, the conditions that favored extraction of oil, also favored extraction of proteins because of the disruption of the oleosome protein membrane. The composition of the oleosome hydrolysates was determined by the urea SDS-PAGE (Fig. 7). Soy flour and isolated defatted oleosomes were used as controls (Lanes 2–3). Surprisingly, proteins from hydrolysate prepared without Protex 6L were hydrolyzed at 4.16 ± 0.17% (Lane 4). This is due to the presence of a protease contaminant in the Multifect pectinase preparation used to fractionate oleosomes from soy flour [40, 41]. Oleosome suspensions prepared with 0.25% Protex 6L for 3–18 h had residual glycinin basic subunit bands (Lanes 6, 7). The suspension prepared with 0.5–2.5% proteases generated peptides smaller than 6.5 kDa (Lanes 1, 8, 9). These results support our findings that 0.5% Protex 6L was the optimum concentration of protease necessary to induce maximal disruption of the oleosome protein membrane.

Conclusion

A promising process for extracting oil from isolated oleosomes was optimized. The optimum conditions consisted of hydrolyzing the oleosome membranes with 0.5% Protex 6L in distilled water at a ratio 1/3; dw/v, pH 9 at 60 °C for 3 h, destabilizing the resulting slurry by heating at 96 °C for 30 min and re-centrifuging of the resulted cream at 10,000×*g* for 15 min. Under these conditions 90% of the free oil was extracted. The free oil recovered presents a similar FA composition to that of the hexane-extracted soybean oil, with an oxidative stability index ranging from 9.06 to 12.03 h. This EAAE process resulted in high quality oil that was 88% lower in free fatty acids compared to hexane-extracted soybean oil. This report is of major importance for finding an efficient alternative to the conventional solvent extraction. In fact, our process as optimized is a simple and environmentally friendly technology

with a process time, labor and energy requirements 56% less than those of solvent extraction [42]. Overall, the raw materials cost is anticipated to be similar to that of Chabrand et al. [42] and would represent only approximately 3% of the production cost.

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