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Enzyme-Assisted Aqueous Extraction of Oleosomes from Soybeans (*Glycine max*)

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Oleosomes, with their unique structural proteins, the oleosins, are known to be useful in cosmetics and other emulsion applications. A procedure to fractionate intact oleosomes to produce soybean oil without the use of organic solvents was investigated. Process parameters, enzyme treatment, filtration, cell lysis, and centrifugation, were studied. Successive extractions of the residue, eliminating the filtration step, pressurization, or ultrasonication of soybean flour prior to enzyme treatment and enzyme treatment on the residue, were the key steps. A mixture of Multifect Pectinase FE, Cellulase A, and Multifect CX 13L in equal proportion gave 36.42−63.23% of the total soybean oil from oleosomes, respectively, for 45 and 180 s of blending time, compared to the conventional method with lower yields (34.24 and 28.65%, respectively, for 45 and 180 s of blending time). Three successive extractions of the residue increased the oil yield to a maximum of 84.65% of the total soybean oil recovered in intact oleosomes. The percentage of lipid in the supernatant fraction decreased to a minimum value of 0.33% with the use of the enzymes at a 3% dosage. The results are considered to be useful for developing large-scale and efficient extraction of oleosomes from soybean.

KEYWORDS: Soybean oleosomes; pectinase; cellulase; hemicellulase; oil recovery

INTRODUCTION

Oleosomes or oil bodies are intracellular plant organelles of about 0.2–2.0 μm in diameter for most oilseeds and 0.2–0.5 μm in the case of soybean (*1–3*). The oleosomes possess a structure consisting of a triacylglycerol core (94–98% of the dry weight) surrounded by a monolayer of phospholipids (0.6–2% w/w) embedded with oleosins and some minor proteins of higher molecular mass (0.6–4% w/w) (*4, 5*). Oleosomes are known to be useful in cosmetics and other emulsion applications (*6, 7*).

Oleosomes can be isolated from hydrated seed homogenates by flotation with centrifugation in a sucrose buffer, as their density is similar to that of oil. The conventional method of extracting oleosomes, involving hydrating intact soybean seeds or cotyledons, grinding, filtering, and centrifuging, is frequently used to fractionate intact oleosomes (*5, 8–10*). Our preliminary investigation determined that the conventional method resulted in intact oleosomes yields of <45% of the total soybean oil. It is obvious that a major contributing factor to the low yield of oleosomes is the inability of the treatment involved to adequately rupture the cellular structure without shearing the oleosome membranes. Therefore, more efficient extraction procedures needed to be developed.

Scanning electron microscopy analysis showed that oleosomes from soybeans are enmeshed in a cytoplasmic network composed of proteins (*11*). The spaces between protein bodies in cotyledon cells are then filled with lipid body and the cytoplasmic network (*1, 12*). Unlike the cytoplasmic features, which are characterized by the presence of protein and lipid, the walls that surround the plant cell are primarily composed of cellulose, hemicellulose, and lignin in addition to pectin. This suggests that enzyme preparations capable of attacking cell walls must contain cellulases, hemicellulases, and pectinases (*13, 14*). Rosenthal et al. (*15, 16*) have reviewed the main aspects relating to aqueous and enzymatic processes for oil extraction and have reported that the disruption of the cell wall of soybean cotyledon during milling operation resulted in an increase in oil and protein extraction yields. Therefore, it could be possible to adapt an enzyme-assisted technique to improve the yield of oleosomes.

The objective of this work was to explore the use of enzymes that hydrolyze cell wall components to improve the release of intact oleosomes from soybean. We compared a conventional method of extracting oleosomes, involving hydrating, grinding, filtering, and centrifuging (*5, 10*), with enzyme-assisted extraction with various modifications in the process to determine the preferable extraction procedure that would enable the release of oleosomes from soybeans.

MATERIALS AND METHODS

Soybeans. Full-fat soybean flour (commodity soybeans of 2006 crop) was procured from the Center for Crops Utilization Research (CCUR),

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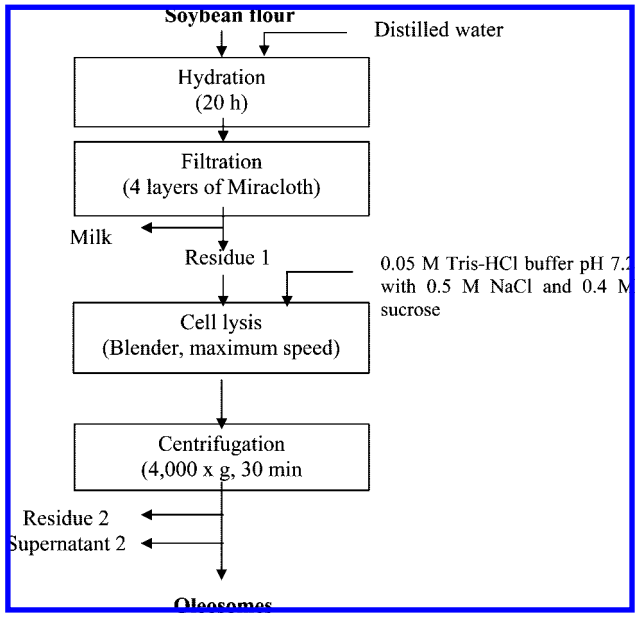

Figure 1. Flow diagram for the extraction of oleosomes by the conventional method modified for dry soy flour.

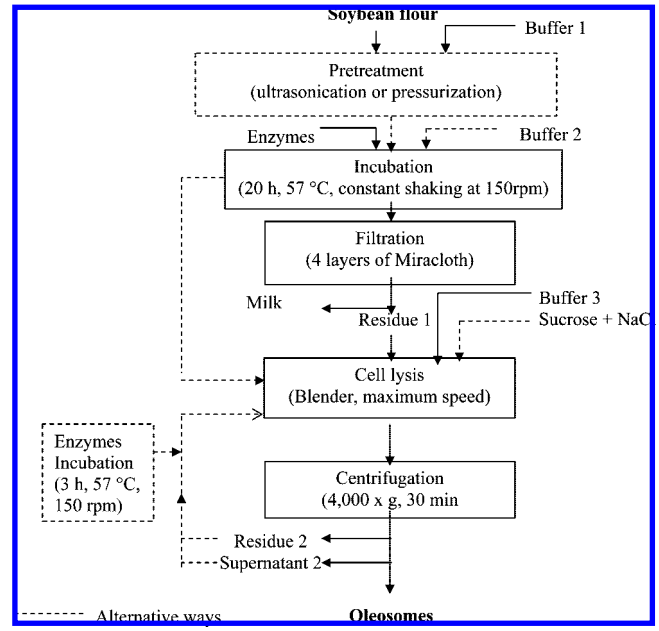

Figure 2. Flow diagram for the enzyme-assisted extraction of oleosomes: buffer 1, 0.1 M potassium acetate buffer, pH 4.6; buffer 2, 0.1 M potassium acetate buffer, pH 4.6, with 0.5 M NaCl and 0.4 M sucrose; buffer 3, 0.05 M Tris-HCl buffer with 0.5 M NaCl and 0.4 M sucrose, pH 7.2.

Iowa State University, Ames, IA. The particle size distribution of soybean flour determined by a laser diffraction particle sizer (Master-Sizer Micro-Plus, Malvern Instruments, Malvern, U.K.) was in the ranges of 0.72–239 μm (88.9%), 275–630 μm (9.56%), and 724–954 μm (1.54%).

Enzymes. Multifect Pectinase FE, Cellulase A, and Multifect CX 13L were used for this study (Genencor International, Inc., Rochester, NY). Multifect Pectinase FE from *Aspergillus niger* is reported to contain pectinase, cellulase, and hemicellulase activities (145–180 units/g). Cellulase A from *Aspergillus* species contains cellulase, β-glucanase, hemicellulase, and xylanase activities. Cellulase A is standardized to an activity of 100 β-glucanase units/gram. Multifect CX 13L is a β-glucanase complex from *Trichoderma reesei* and *Penicillium funiculosum* with a minimum activity of 3900 CMC-DNS units/g (carboxymethylcellulose−3,5-dinitrosalicylic acid unit/g).

All other chemicals and solvents used were of analytical grade.

Conventional Extraction Method. The method was adapted from those of Tzen et al. and Jacks et al. (*5, 10*) and modified as described in **Figure 1**. A weighed quantity of soybean flour was hydrated for 20 h in distilled water in a ratio of 1:6 and filtered through four layers of Miracloth (CalBiochem, La Jolla, CA). The residue was suspended in 150 mL of 0.05 M Tris-HCl, pH 7.2, buffer with 0.5 M NaCl and 0.4 M sucrose and blended with a Waring blender at the maximum speed for 45 s. The homogenate was centrifuged in 500 mL wide-mouth bottles at 4000*g* for 30 min at 4 °C in the swinging bucket rotor JS-4.0 of a Beckman Avanti J-20 series centrifuge (Beckman Instruments Inc., Fullerton, CA) to produce a creamy fat pad (oleosomes) on the surface of the supernatant, the supernatant (milk), and the pellet (residue). The fat pad and the residue were removed with a spatula and freeze-dried. The supernatant fraction was sampled and refrigerated for oil content determination.

Enzyme-Assisted Extraction. The procedure is schematically shown in **Figure 2**. Soybean flour (25 g) was mixed with 150 mL of 0.1 M potassium acetate buffer, pH 4.6. The homogenate was then immediately treated with a mixture of 3% total enzymes separately and in equal proportion (Multifect Pectinase FE, Cellulase A, and Multifect CX 13L), incubated for 20 h at 57 °C with constant shaking at 150 rpm in a Microprocessor Shaker Bath (Melrose Park, IL), and filtered through four layers of Miracloth. The residue on the Miracloth was suspended in 150 mL of 0.05 M Tris-HCl buffer, pH 7.2, with 0.5 M NaCl and 0.4 M sucrose, blended with a Waring blender at the maximum speed, and centrifuged as previously described. The parameters of the process evaluated were as follows: making successive extractions of the residue, eliminating the filtration step, pressurization, or ultrasonication of samples prior to enzyme treatment, and postcentrifugation enzyme treatment of the residue (**Figure 2**).

Extraction on the Residue. The effect of repeated and successive extractions of the residue was investigated. After the first oleosome extraction, the residue was resuspended with the supernatant, blended, and centrifuged as previously described.

High-Pressure as Pretreatment of Samples prior to Oleosomes Extraction. Homogenates (25 g of soybeans flour and 150 mL of 0.1 M potassium acetate buffer, pH 4.6) were sealed under vacuum in plastic bags and pressurized at ambient temperature (25 °C) for 5 min at 200 and 500 MPa using a Food Laboratory 900 Plunger Press System (Stansted Fluid Power Ltd., Essex, U.K.). The sample holder had a 6.5 cm internal diameter and a 23 cm height. A 50% polyethylene glycol solution was used as pressure transmitting fluid. The pressures and temperature of pressurization fluid and vessel were recorded and continuously monitored during the entire period using a Stansted Fluid Power FPG55000 RAP system and a Scan 1000 Supervisory Control and Data Acquisition system (Hexatec, Hexham, U.K.). After pressurization, enzymes were added and the homogenates were incubated, blended with sucrose (20.52 g) and sodium chloride (4.38 g), and centrifuged as previously described (**Figure 2**). The residue was resuspended with the supernatant, blended, and centrifuged, and a second extraction of oleosomes was performed.

Ultrasonication as Pretreatment of Samples prior to Oleosome Extraction. Homogenates (25 g/150 mL of 0.1 M potassium acetate buffer, pH 4.6) were sonicated at room temperature using a 480 W Blackstone ultrasonic probe (Blackstone Ultrasonics Inc., Sheffield, PA) 70 W for 3, 5, and 10 min. After ultrasonication, enzymes were added and the homogenates were incubated, blended with sucrose (24.54 g) and sodium chloride (4.38 g), and centrifuged as previously described (**Figure 2**). The residue was resuspended with the supernatant, blended, and centrifuged, and a second extraction of oleosomes was performed.

Enzyme Incubation in the Residue. This procedure included enzyme treatment with 0.1 M potassium acetate buffer, pH 4.6, with 0.4 M sucrose and 0.5 M NaCl, incubation, blending, centrifugation, incubation of residue resuspended in the supernatant for 3 h, blending, and centrifugation (**Figure 2**).

Light Microscopy. Small droplets of fat pad were placed between a slide and a coverslip of a BX-51 Olympus light microscope (magnification 100×). Images were acquired using a Pixel ink digital camera (Olympus, Tokyo, Japan) attached to the microscope.

Oil Recovery and Protein Determination. Oil was extracted from the freeze-dried oleosomes with hexane, using a Goldfisch apparatus (*17*). In the milk (supernatant), the AOAC (*18*) method was used. The residual oil in the pellet (residue) was also determined by Goldfisch (*17*). The amounts of oil recovered were calculated as percentage of total oil present in unprocessed soybean flour. The solvent extraction gave a yield of 24.49 ± 0.15 g of oil/100 g, $n = 3$, of soybeans. A value of 24.49 ± 0.15 g of oil/100 g of soybeans was taken as 100% when the oil recovered from oleosomes was calculated. Protein contents of pressurized sample were determined with the Dumas method by using a rapidN III Nitrogen Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ) (*19*). The protein conversion factor was 6.25.

Statistical Analysis. The general linear model, PROC GLM, in SAS system (version 8.2, SAS Institute, Inc., Cary, NC) was used to compare the means at $P < 0.05$. All experiments were done in triplicate.

RESULTS AND DISCUSSION

Conventional Method. The conventional method of Tzen et al. and Jacks et al. (*5, 10*), developed for seed cotyledons, was modified for dry soy flour and used to extract soybean oleosomes (**Figure 1**). The total bean oil recovered from oleosomes was 34.28 ± 2.57% for the sample processed in Waring blender for 45 s. As the blending time was raised to 180 s, the total soybean oil recovered from oleosomes decreased to 28.65 ± 2.61%. The oil yield difference was significant at $P < 0.05$. Increasing the blending time created an emulsion in the supernatant after centrifugation. The oils recovered from the supernatant fraction were 17.95 ± 0.12 and 30.82 ± 2.06%, respectively, for samples blended with a Waring blender for 45 and 180 s, and the difference was significant at $P < 0.05$. These results are in agreement with our preliminary investigation on extraction of oleosomes from soybean cotyledons, which showed that oleosomes extracted by the conventional method contain <45% of the total soybean oil. The low yield obtained with theTzen et al. and Jacks et al. (*5, 10*) conventional method has necessitated the search for various modified protocols. The use of enzymes, mechanical disruption of cell wall, extraction on the residue, and pretreatment of the samples have been evaluated.

Enzyme-Assisted Extraction Method without Pretreatment. This procedure involved enzyme treatment, filtration, blending, and centrifugation; the total soybean oil recovered from oleosomes was 36.42 ± 1.80, 44.68 ± 1.32, and 63.61 ± 3.41% when the homogenates were blended for 45, 90, and 180 s, respectively, with the use of a mixture of enzymes in equal proportion (Multifect Pectinase FE, Cellulase A, and Multifect CX 13L, 1% of each for a total of 3% v/w). Blending the homogenate for 360 s did not increase the yield of oil recovered from oleosomes. Microscopic observation carried out showed intact oil bodies in the fat pad (**Figure 3**).

Because a 180 s homogenization gave the best yield, further experiments were carried out with 180 s of blending time. The effects of enzyme concentration and successive extraction of the residue were investigated. In the initial extraction, the total soybean oil extracted from oleosomes was sufficiently high: 38.19 ± 0.98, 52.34 ± 0.34, 58.64 ± 1.38, and 63.23 ± 3.41% were obtained with the use of 0, 0.6, 1.5, and 3% v/w (volume of enzyme per weight bean), respectively, of total enzymes (**Figure 4A**). The second extraction of the residue made it possible to get 6.63−14.13% of the total soybean oil as oleosomes. The third extraction gave 2.04−4.97%. By the fourth extraction on the residue, 1.32−2.56% yields were obtained. The summation of the yield in these four steps accounted for 48.97 ± 4.51, 57.19 ± 1.02, 82.04 ± 2.81, and 84.65 ± 1.46% of the total soybean oil from oleosomes, respectively, with 0, 0.6, 1.5 and 3% total enzymes. A two-step extraction appeared to release most of the soybean oleosomes, providing an efficient and economical process.

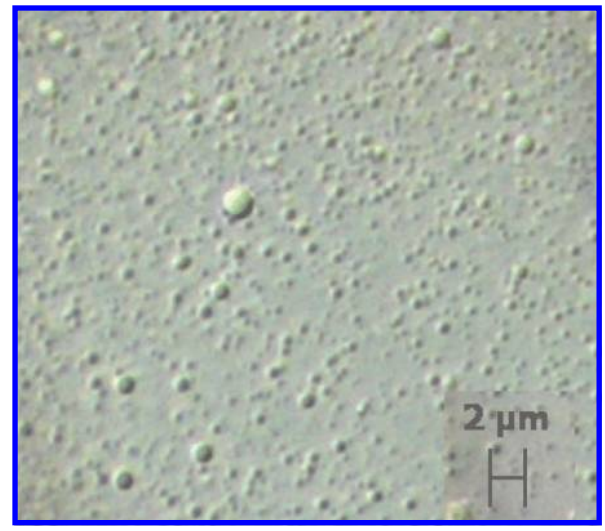

Figure 3. Light microscopy of oleosomes at a magnification factor of 100×.

Oils in the supernatant fraction significantly ($P < 0.05$) decreased with increasing enzyme concentration (**Table 1**). However, a fraction of oil remains trapped in the cellular matrix. Oils in the residue were extracted using the classical hexane extraction method. The results showed that the lowest yield (2.19%) of oil in the residue was with the use of 3% of enzyme by the procedure involving three successive extractions in the residue (**Table 1**).

Further optimization of the process was conducted by analyzing the effect of eliminating the filtration step, evaluating the use of ultrahigh pressurization of soybeans flours or ultrasonication of soybeans flours prior to enzyme treatment, and enzyme treatment on the postcentrifugation residue.

Enzyme-Assisted Extraction Method with Pretreatment prior to Incubation. The ultrahigh pressurizations of soybeans flours were done at 200 and 500 MPa for 5 min at 25 °C. Pressurized samples were then incubated with 3% total enzymes or without enzyme for 20 h, with constant shaking at 150 rpm. The data presented in **Figure 4B** show the highest yield of 55.68 ± 4.36% was obtained with the sample pressurized at 200 MPa and subsequently incubated with 3% total enzymes. After a second extraction on the residue, this yield reached 71.39 ± 2.68%. With samples pressurized at 500 MPa, the yields of oil recovery were 7.16 ± 1.15 and 21.82 ± 0.49%, respectively, with 0 and 3% total enzymes. The effect of high pressure on functional properties had been studied by Butz et al. for some vegetables (*20*). They discovered that in most cases, a pressure around 600 MPa induced changes in the structure of the products which resulted in altered physicochemical properties such as water retention or reduced extractability. Ahmed et al. (*21*) found that egg albumen coagulates completely and irreversibly at 700 MPa. From the data in **Figure 4B**, it is interesting to note the total bean oil recovered from oleosomes is 51.74 ± 2.63% for samples pretreated at 200 MPa and incubated with 0% of enzyme, which is higher than the yield of initial extraction procedure without the use of enzymes.

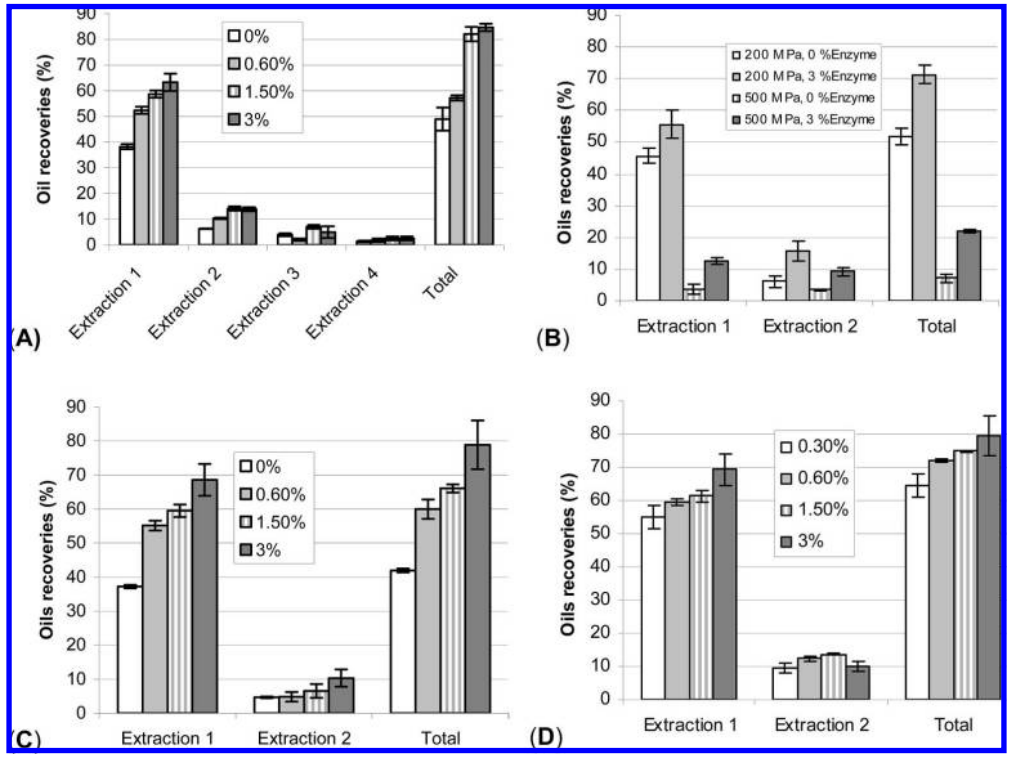

Figure 4. Effects of enzyme concentration on the total soybean oil recovered from oleosomes: (**A**) procedure involving incubation with 0.1 M potassium acetate, filtration, blending with 0.05 M Tris-HCl buffer with 0.5 M NaCl and 0.4 M sucrose, centrifugation, and three successive extractions in the residue; (**B**) procedure involving pressurization of soybean flour prior to enzyme treatment; (**C**) procedure involving 3 min of ultrasonication of soybean flour prior to enzyme treatment; (**D**) procedure involving enzyme treatment in the residue.

Table 1. Residual Oil (Dry Weight Basis) Recovered from the Supernatant Fraction and Residue by the Enzyme-Assisted Extraction[a]

enzyme concentration (%)	oil in milk (%)	oil in the supernatant fraction 2 (%)	oil in residue (%)
Procedure Involving Incubation with 0.1 M Potassium Acetate, Filtration, Blending with 0.05 M Tris-HCl Buffer with 0.5 M NaCl and 0.4 M Sucrose, Centrifugation, and Three Successive Extractions in the Residue			
0	0.08 ± 0.02	15.05 ± 1.54d	8.08 ± 0.75c
0.6	0.10 ± 0.01	6.24 ± 0.78c	5.38 ± 0.60b
1.5	0.08 ± 0.01	1.41 ± 0.32b	5.29 ± 0.66b
3	0.012 ± 0.01	0.33 ± 0.02a	2.19 ± 0.89a
Procedure Involving Pressurization of Soybean Flour prior to Enzyme Treatment			
0 (200 MPa)		11.52 ± 1.16d	6.32 ± 2.12a
3 (200 MPa)		2.17 ± 0.20a	6.56 ± 2.59a
0 (500 MPa)		4.16 ± 0.82b	66.48 ± 5.18c
3 (500 MPa)		8.16 ± 0.68c	51.75 ± 5.28b
Procedure Involving 3 min of Ultrasonication of Soybean Flour prior to Enzyme Treatment			
0		12.30 ± 1.14d	5.94 ± 0.50a
0.6		7.92 ± 0.97c	4.42 ± 0.83a
1.5		4.40 ± 0.09b	3.75 ± 0.43a
3		3.19 ± 0.60a	4.43 ± 0.53a
Procedure Involving Enzyme Treatment in the Residue			
0.3		9.75 ± 0.34c	6.05 ± 0.60a
0.6		6.72 ± 1.15b	6.63 ± 1.57a
1.5		4.45 ± 0.48a	5.49 ± 0.43a
3		4.27 ± 0.19a	5.39 ± 0.58a

[a] Values are mean ± SD ($n = 3$). Values with different letters within each column for each procedure differ significantly ($P < 0.05$).

Residual oils in the supernatant fraction and the residues are shown in **Table 1**. Samples pressurized at 500 MPa resulted in most of the oil (56.75−66.48% of the total soybean oil) remaining in the residue. To better understand the differences in extraction yields, we determined protein content of the extract with the Dumas method by using a Rapid N III Nitrogen Analyzer. Total soybean proteins in the supernatant fraction were significantly lower in samples pressurized at 500 MPa (24.67 ± 1.13 and 27.13 ± 0.43 for sample treated with 0 and 3% total enzyme, respectively) compared to samples pressurized at 200 MPa (38.97 ± 2.51 and 50.36 ± 1.13 for sample treated with 0 and 3% total enzyme, respectively) and to samples not pressurized (48.20 ± 0.25 and 56.76 ± 0.56 for samples treated with 0 and 3% total enzyme, respectively). Higher values of total soybean protein were obtained in the residue of samples pressurized at 500 MPa (66.05 ± 2.81 and 52.17 ± 3.63 for samples treated with 0 and 3% total enzyme, respectively) compared to samples pressurized at 200 MPa (26.07 ± 1.96 and 16.71 ± 0.23 for sample treated with 0 and 3% total enzyme, respectively) and to samples not pressurized (23.18 ± 1.33 and 14.39 ± 0.52 for sample treated with 0 and 3% total enzyme, respectively). Similarly, Lakshmanan et al. (*22*) reported that the protein solubility of soy milks decreased after pressure treatment at 500 and 600 MPa regardless of the pH and soy-to-water ratio.

Experiments were conducted to study the effect of ultrasonication pretreatment on the total bean oil recovered from oleosomes. The flour samples were sonicated for 3, 5, and 10 min at 70 W, and the extractions were made with 3% total enzymes. The highest yield was obtained with for samples sonicated for 3 min (78.87 ± 7.19%). The yield of total soybean oil recovered from oleosomes decreased with the increase of the time of ultrasonication (66.50 ± 2.33 and 63.39 ± 2.89, respectively, for 5 and 10 min). Although ultrasound pretreatment prior to enzyme treatment induced a better contact between damaged cell walls and enzyme in the extraction medium, ultrasonic energy is not homogeneously distributed during the pretreatment (*23, 24*). Experiments were conducted to study

the effect of enzyme concentration on the extraction yields. The extraction was performed on samples sonicated for 3 min at 70 W, because 3 min induced a slightly better recovery. The yields of total soybean oil recovered from oleosomes significantly ($P < 0.05$) increased with the increase of enzyme concentration and were 41.90 ± 0.54, 59.97 ± 2.84, 66.06 ± 1.22, and 78.87 ± 7.19%, respectively, with 0, 0.6, 1.5, and 3% total enzymes (**Figure 4C**). Total soybean oils in the supernatant fraction and residues were in the ranges from 3.19 ± 0.60 to 12.30 ± 1.14 and from 3.75 ± 0.43 to 5.94 ± 0.50, respectively (**Table 1**).

With the procedure involving enzyme incubation with 0.1 M potassium acetate, pH 4.6, prepared with 0.4 M sucrose and 0.5 M NaCl, the yields of total soybean oil recovered from oleosomes were 54.92 ± 3.41, 59.46 ± 1.05, 61.33 ± 1.65, and 69.34 ± 4.75% after the first extraction, with 0.3, 0.6, 1.5, and 3% of enzyme, respectively (**Figure 4D**). Enzyme treatment of the residue for 3 h at 57 °C with constant shaking at 150 rpm made it possible to get 10.00 ± 1.43, 12.48 ± 0.74, 13.59 ± 0.19, and 10.11 ± 1.55%, respectively, with 0.3, 0.6, 1.5, and 3% total enzyme. The summation of the yields in these two steps accounted for a maximum value of 79.45 ± 6.13%. Total soybean oils in the supernatant fraction and residues were in the range from 4.27 ± 0.19 to 9.75 ± 0.34% and from 5.39 ± 0.58 to 6.63 ± 1.97%, respectively (**Table 1**).

Comparison of Different Methods. The selection of an oleosome extraction method would mainly depend on the advantages and disadvantages of the processes, such as extraction yield, complexity, production cost, environmental friendliness, and safety. The conventional methods of Tzen et al. and Jacks et al. (*5, 10*) are the most frequently used extraction procedures for oleosome isolation because they are simple and yield purified oleosomes. The drawbacks of these methods are the low extraction efficiency and the increase of emulsion formation. In general, aqueous enzymatic extraction gives higher oil extraction yields from oleosomes. With the pressurization prior to extraction of oleosomes, the pressure used determines the extraction yields. The extraction of oleosomes on low pressurized samples has the advantage to give high yields without the use of enzyme. However, high-pressure processing is still costly, mainly because of the initial capital expenditure, and may limit this application. Ultrasonication prior to enzyme treatment showed that ultrasonic pretreatment gives high yield when it is used for 3 min. The drawback of this procedure is the fact that ultrasonic energy is not homogeneously distributed and could induced low precision. The drawback of the second enzyme treatment in the residue is the large amount of enzymes needed. Considering the expensive enzyme consumption, the long extraction period, and the yields of oleosomes, these alternative procedures are not favorable from a commercial perspective. The enzyme-assisted procedure with several extractions on the residue mainly depends on the enzyme concentration combined to the mechanical disruption of the cell residue to obtain the maximum yield. In general, this procedure is user-friendly and gives high extraction yield. The drawback of this procedure could be the fact that it is labor-intensive. However, compared to the procedures with a second enzyme treatment of the residue and compared to the procedures with pressurization or ultrasonication prior to enzyme treatment, the enzyme-assisted procedure with several extractions on the residue showed high extraction efficiency. Furthermore, the procedure suitable for oleosome extraction required simple equipment that can be operated safely. Additionally, our proposed enzyme-assisted process fits into the soy protein isolate technology in the food industry. Undenatured soy protein and/or the soy storage proteins glycinin and β-conglycinin can be recovered from the aqueous supernatant in addition to isolating the oleosome fraction (preliminary data not shown).

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