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ORIGINAL PAPER

Recycling of Aqueous Supernatants in Soybean Oleosome Isolation

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Abstract Oleosome extractions from soybean flour typically generate significant quantities of aqueous sucrose- and sodium chloride-rich supernatant which could be recycled. To determine the feasibility of recycling the oleosome process aqueous supernatants, three extraction protocols were evaluated. The first extraction used the original extraction solution, 0.1 M fresh potassium acetate pH 4.6 containing 0.4 M sucrose and 0.5 M NaCl. The second protocol reused the aqueous supernatant obtained from the first extraction. The third protocol reused the aqueous supernatant obtained from the second protocol. Oleosome extraction yields were significantly higher in the first extraction with enzymes (Multifect® Pectinase FE, Multifect® GC, and Multifect® CX B, 1% each, v/w) compared to the yield when the supernatant was reused with no additional enzymes (81.41 ± 2.24 vs. 73.09 ± 3.39%, respectively). Oil yields from oleosome fractions were not statistically different when extractions were made with 0 or 3% enzymes in the third protocol. Protein was the predominant constituent in the supernatant in addition to mineral and carbohydrate. Soybean storage protein profile from recycled supernatants obtained without adding enzyme were similar to a traditional soy protein water extract but with a decrease of intensity of the β-conglycinin bands. Addition of 3% enzymes in both recycling protocols resulted in the disappearance of the α' and α subunits of the β-conglycinin due to a protease contaminant in Multifect® Pectinase FE. Results from this work revealed essential information for a promising possibility of the future industrial application of this technology.

Keywords Oleosome extraction · Recycling · Enzymes · β-Conglycinin · Glycinin

Introduction

Oleosomes are small, discrete subcellular organelles in which plant seeds store triacylglycerol and can be isolated to provide important functional ingredients to the food, cosmetic and pharmaceutical industries. The search for more efficient means of extracting oleosomes of both higher quality and yield has led to the development of variety of protocols [1–6]. However, the fundamentals of oleosomes extraction remain the same. Oleosomes must be isolated by flotation with centrifugation from cellular material. A high osmotic, high ionic strength buffer prevents disruption of the organelles' membrane. Interestingly, isolation of oleosomes is based on the green aqueous extraction processing that is particularly efficient with the use of enzymes for simultaneous extraction of both oil and undenatured protein [7].

A recent study [6] suggested that increasing the yield of oil recovery in oleosomes is feasible with the use of enzymes combined with the mechanical disruption of cell walls. This procedure resulting in high yield of oleosomes from soybeans has been demonstrated on a pilot plant scale, using a continuous flow operation three-phase decanter and could lead the process to be used industrially. We have shown that, from 75 kg of starting soybean flour, oleosomes isolation at a pilot plant scale generates at least 200 L of the aqueous solution. Consequently, the industrial production of oleosomes will typically generate significant

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quantities of aqueous supernatant, with high salt and sucrose content. This important fraction, if considered as waste, would increase the production costs due to the high concentrations of sucrose and sodium chloride thus, could produce environmental impacts.

No studies to date appear to have evaluated the potential of recycling the aqueous supernatant used for oleosome extraction. Furthermore, no studies describe the characteristics of aqueous supernatant when managed as a by-product. Therefore, the aim of this study was to investigate the reuse of aqueous supernatant from a primary extraction and from a first protocol of recycling to isolate oleosomes from soybean flour. Some characteristics of these supernatants were evaluated by determining the following constituents: oil, protein, ash, carbohydrate and storage protein peptide profile.

Materials and Methods

Materials

Full-fat, dehulled soybean flour from variety NK25D3 harvested in 2007 was obtained from Natural Products Inc (Grinnell, IA, USA). The flour contained 6.70% moisture, 39% (d.b.) protein, 31.40% (d.b.) lipid and 4.90% (d.b.) ash. The particle size distribution of soybean flour determined by a Mastersizer-2000 particle size analyzer (Malvern Instruments Ltd, Worcestershire, UK) with a wet module (Hydro 200) was in the ranges 0.63–104.71 μm (71.29%), 120.22–478 μm (26.05%) and 549.54–954.99 μm (2.65%).

All enzymes, Multifect® Pectinase FE (E.C. 4.2.2.10, pectinase, optimal pH 3.85, optimal temperature 45 °C), Multifect® GC (E.C. 3.2.1.4, cellulase, optimal pH 4.0, optimal temperature 55 °C), Multifect® CX B (E.C. 3.2.1.4, cellulase complex standardized on β-glucanase, optimal pH 5.0, optimal temperature 40 °C) were provided by Genencor International (Rochester, NY, USA). The protease inhibitor cocktail for yeast and fungi (Cat P8215—5 mL, Lot 037K4018), used to verify the presence of protease side activity in the enzyme preparation, was from Sigma–Aldrich (St Louis, MO, USA).

All chemicals and solvents used were of analytical grade and were from Sigma (St Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Extraction Procedure

The primary extraction was performed by mixing 2.5 kg of soybean flour and 0.1 M fresh potassium acetate buffer pH 4.6 containing 0.4 M sucrose and 0.5 M NaCl in a flour to a buffer ratio of 1:6. An equal proportion of Multifect® Pectinase FE, Multifect® GC, and Multifect® CX B equaling 3% (v/w) total enzymes was added immediately and the homogenate was incubated for 16 h at 57 °C in a 20-L process reactor (model CG-1965-620; ChemGlass Inc., Vineland, NJ, USA), at 150 rpm. The homogenate was then blended for 3 min with a Waring blender, and centrifuged as described previously [6]. Three fractions were obtained: oleosomes; residue; and an aqueous supernatant, which was reused in the second and third protocols.

Recycling of Aqueous Supernatant for Oleosome Extraction

The recycling of aqueous supernatant used to isolate oleosomes and recover undenatured soybean protein is important for future industrial application of this technology. Two protocols of extractions were carried out to study the reuse of aqueous supernatant from a primary extraction. In the first protocol, the primary supernatant (PS) obtained from the extraction of oleosomes using soybean flour and 0.1 M fresh potassium acetate buffer pH 4.6 containing 0.4 M sucrose and 0.5 M NaCl was adjusted to pH 4.6 and reused to extract oleosomes from fresh soybean flour. In the second protocol, supernatant obtained from the first protocol were adjusted to pH 4.6 and reused to isolate oleosomes from fresh soybean flour. Experiments were performed using 1:6 soybean flour-to-supernatant ratios, with either 0 or 3% of enzymes mixture to collect oleosomes, supernatant and residue as previously described.

Analytical Methods

The following constituents were determined on oleosome extracts, supernatants and residues: total oil contents were determined in the oleosome fractions and the residues by hexane B extraction using a Goldfisch apparatus (Labconco, Kansas city, MO, USA) [8]. In the supernatants, the Mojonnier method was used [9]. Ash content was determined by igniting the samples at 550 °C in a Thermolyne muffle furnace (Labline instruments, Melrose Park, IL, USA) until light gray ash resulted. The protein contents of solid and liquid fractions were determined by using a combustion-type nitrogen analyzer (Elementar Americas, Mt. Laurel, NJ, USA) for the Dumas method [10]. The nitrogen values were multiplied by 6.25 to estimate the protein contents. Carbohydrate was determined by difference. To monitor the degradation of the cell wall components after enzyme treatment, reducing sugars production from soybean flour in the aqueous supernatant were quantified by the 3,5-dinitrosalicylic acid (DNS) method [11] using a Genesis 2 spectrophotometer (Spectronic-Unicam, Rochester, NY, USA).

Recoveries

The recoveries were expressed as percentages of each component in each fraction relative to the initial amounts in the soybean flour and the aqueous supernatant used on a dry weight basis.

Ultrafiltration

An Amicon stirred cell module (Millipore, MA, USA) fitted with a 1-kDa Ultracel regenerated cellulose membrane disc with a diameter of 63.5 mm was used in ultrafiltration experiments. Each supernatant, 90 mL, was filtered until the volume of the retentate decreased to approximately 5 mL. Prior to electrophoresis, the protein content of the retentate was determined by Biuret assay using bovine serum albumin (BSA) as a reference protein [12].

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the retentate was performed using a gradient gel according to the method of Laemmli [13]. A wide-range molecular weight marker (6.5–205 kDa) for SDS-PAGE was used to determine the molecular weights. For quantification of the glycinin and β-conglycinin fractions and their respective subunits, the gels were rinsed after destaining, scanned with an Amersham Pharmacia Biotech Image Scanner (Piscatawa, NJ, USA) and the protein bands subjected to Densitometric analysis with a Kodak molecular imaging software version 4.05f5.

Statistical Analysis

All analyses and treatments were carried out in triplicate. SAS software (version 9.1.2, 2004; SAS Institute Inc., Cary, NC) was used for analysis of variance (ANOVA). Statistical significance was determined at the $P < 0.05$ level.

Results and Discussion

Primary Extraction

The objective of this study was to determine the ability of recycled aqueous supernatant to isolate oleosomes. The primary extraction was performed as described under "Materials and Methods", using soybean flour and 0.1 M fresh potassium acetate buffer pH 4.6 containing 0.4 M sucrose and 0.5 M NaCl, and a 3% of enzymes mixture. Figure 1 shows the percentage of oil, protein, ash and carbohydrate recoveries in the oleosome, supernatant and residue fractions for this primary extraction. The recoveries in the oleosome fraction were 81.64 ± 1.16% oil, 35.92 ± 0.52% protein, 17.82 ± 0.16% ash, and 19.84 ± 0.38% carbohydrate of total soybean oil, protein, ash, and carbohydrate, respectively. There was no significant difference compared to the oil extraction yield (84.65 ± 1.46%) reported in our earlier report [6]. The supernatant fraction contains mainly protein, mineral and carbohydrate. The recoveries were 52.14 ± 0.05, 70.79 ± 0.69, and 67.06 ± 2.44, respectively, for protein, ash and carbohydrate. The oil yield was low in the supernatant fraction (2.46 ± 0.0%), as expected.

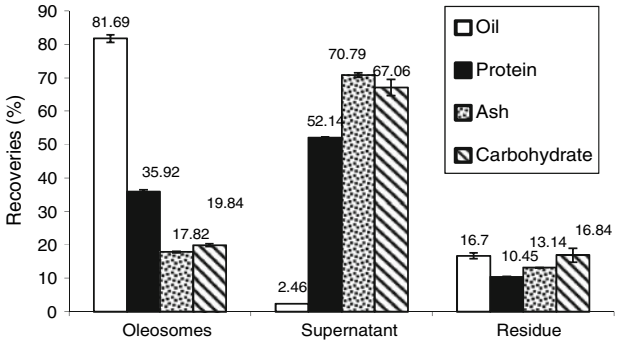

Fig. 1 Oil (*open bars*), protein (*solid bars*), ash (*stippled bars*) and carbohydrate (*hatched bars*) recoveries in the oleosomes, supernatant and residue fractions after the primary extraction

Recycling the Aqueous Supernatant for Oleosomes Extraction

To determine whether the supernatant could be reused, fresh full fat soybean flour was incubated with the PS from a previous extraction, with either 0 or 3% enzymes (cellulase/pectinase) treatment, and oleosomes were isolated as previously described. This procedure was performed for two protocols of extractions.

Figure 2 shows the oil, protein, ash and carbohydrate distribution in the various fractions when recycling the supernatant from the primary extraction with either a 0 or 3% enzymes treatment. Total soybean oil recovered from oleosomes is 73.09 ± 3.39% for samples incubated with 0% of enzyme. It should be noted that the PS was obtained after an initial extraction performed with the 3% enzymes treatment. Thus the PS recycled with the 0% enzyme treatment in this first reuse protocol contained residual enzymes activities from the initial extraction. As seen in Fig. 2, the addition of 3% enzymes when using the PS in the first recycling protocol significantly increases the extraction yield of oleosomes (81.41 ± 2.24%). On the other hand, it is clear that the percentages of protein, ash and carbohydrate recovered in the aqueous supernatant

were lower when the PS was reused with 3% enzymes compared to the recoveries when the PS was used with 0% enzyme treatment (Fig. 2). The increase in protein, ash and carbohydrate contents followed by a reduction in their recoveries when the PS was reused with 3% enzymes is related to the appreciable increase of the oleosome fraction obtained and the reduction of the volume of aqueous supernatant after oleosome extraction.

Oil, protein, ash and carbohydrate recoveries of different extracts after a second recycling of the aqueous supernatant with fresh flour are shown in Fig. 3. The yields of oil recovered from oleosomes were not statistically different when the extraction was made either with a 0 or 3% enzymes treatment. The highest yield was 77.66 ± 1.29% of total soybean oil recovered from oleosomes (Fig. 3). Protein, ash and carbohydrate recoveries in the second recycle phase of the supernatant were lower compared to the first recycle series.

Chemical Characteristics of Aqueous Supernatants

The oil, protein, ash, reducing sugars and carbohydrate means are presented in Table 1. The main components of the initial buffer were potassium acetate (0.1 M) sucrose (0.4 M) and sodium chloride (0.5 M). Using this buffer to fractionate oleosomes from soybean flour generated a large volume of supernatant. Some characteristics of aqueous supernatant recycled for two consecutive series of oleosome extraction show that oil content in the PS was only a trace (0.15 g/100 mL). Reusing the PS in the first protocol of extractions shows that oil contents were 0.55 and 0.30 g/100 mL when the supernatant was reused with 0 and 3% enzymes, respectively. The second recycling protocol of the aqueous supernatant shows that the lowest value of oil content (0.10 g/100 mL) was obtained when enzymes were added. This suggested that adding 3% enzymes when reusing the supernatant resulted in a considerable oil release from the aqueous supernatant. Since the oleosomes survived intact and floated to the oil layer, oil in the supernatant was probably the result of contamination that could occur when removing the oleosome fraction. Lamsal and Johnson [14] reported that a cocktail of enzymes can achieve a similar effect in the soybean emulsion cream after their aqueous extraction process. Ash and carbohydrate contents did not change when the supernatant were reused in two consecutive protocols with either 0 or 3% enzymes.

Reducing sugar contents were determined in the aqueous supernatants obtained after two recycling protocols with or without adding enzyme (Table 1). The reducing sugar content in the PS increased from 6 mg/g soybean flour for the control (primary extraction with no enzymes) to 115.56 mg/g soybean flour, indicating that the cellulases and pectinases partially hydrolyzed the cell wall components. The consecutive reuse of PS in two protocols of extraction with 0% enzyme indicates that the cellulases and pectinases have been active, and results in more reducing sugars. On the other hand, reducing sugar production increased when the aqueous supernatant was reused with a 3% enzymes mixture in the first and the second protocols.

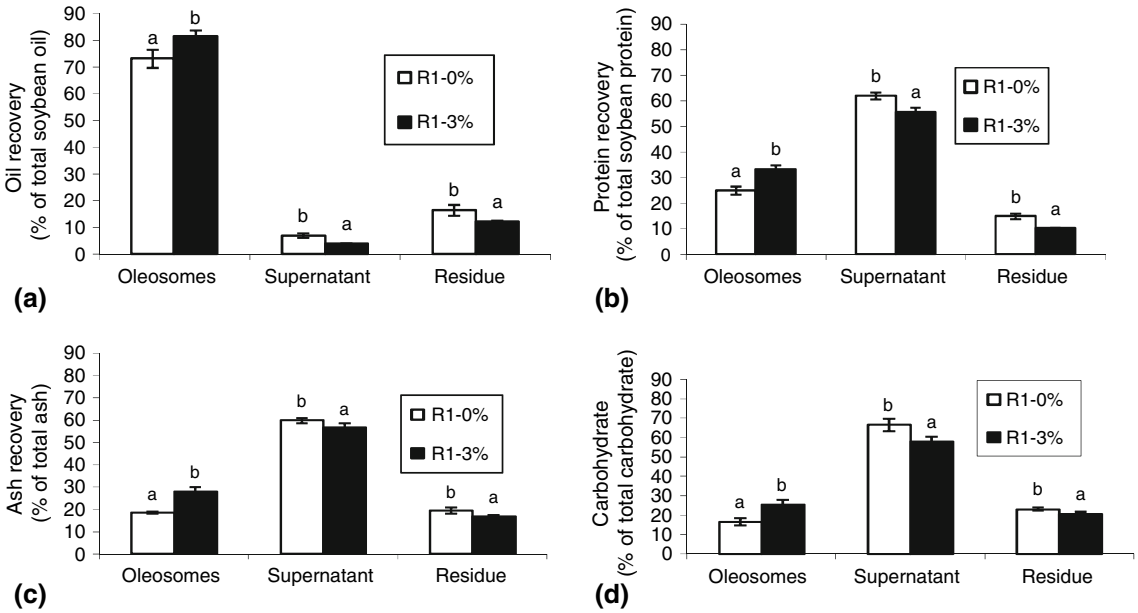

Fig. 2 Oil (**a**), protein (**b**), ash (**c**), and carbohydrate (**d**) recoveries in oleosomes, supernatant and residue fractions after recycling the primary supernatant with no enzymes (R1-0% *open bars*) or with 3% enzymes (R1-3% *filled bars*). Fractions sharing the same letter for each recovery are not significantly different at $p < 0.05$. Carbohydrate was determined by difference

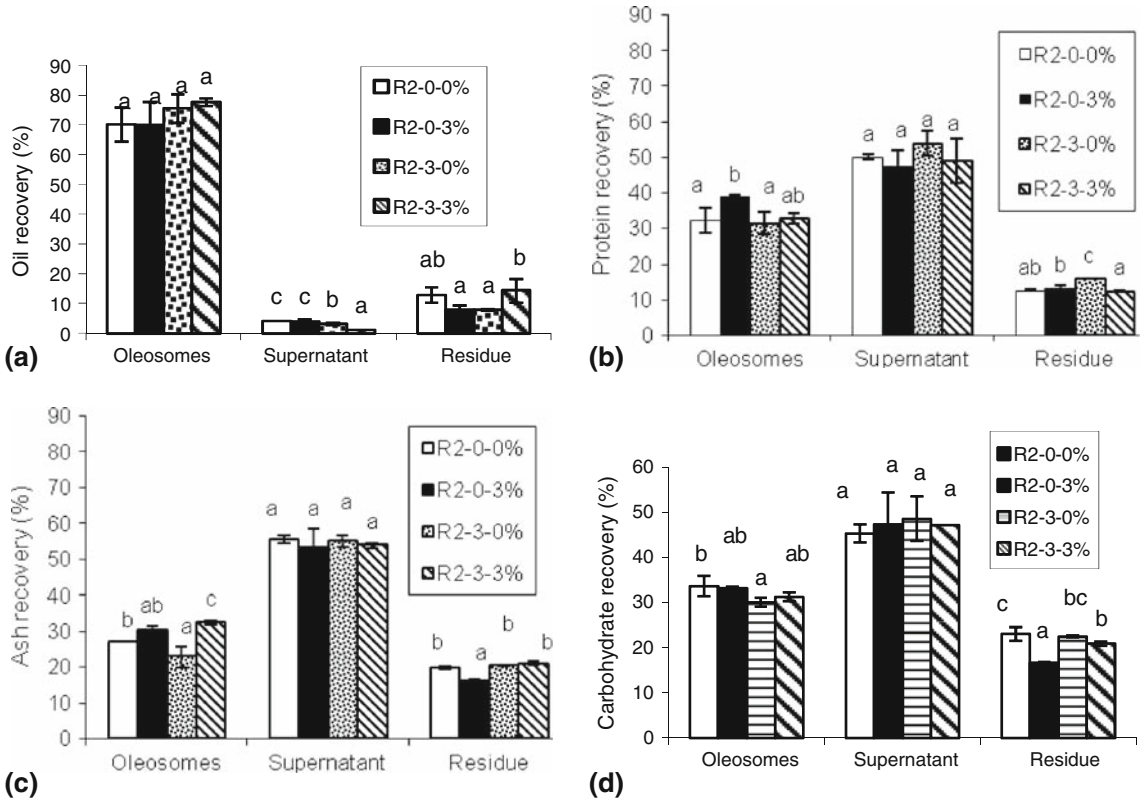

Fig. 3 Oil (**a**), protein (**b**), ash (**c**) and carbohydrate (**d**) recoveries in oleosomes, supernatant and residue fractions after a second recycling of the aqueous supernatant: R2-0-0% (supernatant obtained after reusing R1-0% with no enzymes, *open bars*), R2-0-3% (supernatant obtained after reusing R1-0% with 3% enzymes, *solid bars*), R2-3-0% (supernatant obtained after reusing R1-3% with no enzymes, *stippled bars*), R2-3-3% (supernatant obtained after reusing R1-3% with 3% enzymes, *hatched bars*). Carbohydrate was determined by difference

Table 1 Moisture, lipid, protein, ash, carbohydrate and reducing sugar contents of aqueous supernatant obtained after reusing the supernatant with 0 or 3% enzymes

Parameters	PS	First recycling protocol		Second recycling protocol			
		R1-0%	R1-3%	R2-0-0%	R2-0-3%	R2-3-0%	R2-3-3%
Moisture (%)	77.45 ± 0.07^{d}	72.15 ± 0.54^{c}	73.67 ± 0.34^{c}	67.49 ± 0.07^{b}	68.28 ± 1.28^{b}	67.07 ± 1.68^{ab}	65.11 ± 0.12^{a}
Lipid (g/100 mL)	0.15 ± 0.07^{a}	0.55 ± 0.07^{c}	0.30 ± 0.00^{b}	0.35 ± 0.07^{b}	0.30 ± 0.10^{b}	0.25 ± 0.07^{b}	0.10 ± 0.00^{a}
Protein (g/100 mL)	4.10 ± 0.35^{a}	8.97 ± 0.29^{b}	8.36 ± 0.29^{b}	11.64 ± 0.01^{c}	11.60 ± 0.03^{c}	12.58 ± 0.51^{d}	11.33 ± 0.03^{c}
Ash (g/100 mL)	4.86 ± 0.05^{d}	4.31 ± 0.03^{b}	4.23 ± 0.04^{a}	4.76 ± 0.03^{c}	4.87 ± 0.06^{cd}	4.77 ± 0.07^{cd}	4.92 ± 0.06^{d}
Carbohydrate (g/100 mL)	13.42 ± 0.41^{a}	14.20 ± 1.06^{a}	13.43 ± 0.04^{a}	15.75 ± 0.12^{a}	14.91 ± 1.13^{a}	15.31 ± 2.01^{a}	18.65 ± 0.11^{b}
Reducing sugars (mg/g soybean flour) after enzyme incubation	115.56 ± 2.15^{b}	100.78 ± 14.66^{b}	163.90 ± 7.85^{c}	86.72 ± 03.60^{a}	155.71 ± 14.20^{c}	211.00 ± 5.40^{d}	253.20 ± 0.90^{e}

Means in rows with different superscript letters are significantly different at $P < 0.05$

PS supernatant obtained from an initial extraction, after using soybean flour, buffer and 3% of enzyme. *R1-0%* supernatant obtained after reusing PS with no enzymes, *R1-3%* supernatant obtained after reusing PS with 3% enzymes, *R2-0-0%* supernatant obtained after reusing R1-0% with no enzymes, *R2-0-3%* supernatant obtained after reusing R1-0% with 3% enzymes, *R2-3-0%* supernatant obtained after reusing R1-3% with no enzymes, *R2-3-3%* supernatant obtained after reusing R1-3% with 3% enzymes

The higher the enzyme concentration, the greater the hydrolysis of the cell wall components. However, the addition of enzymes continuously in the first and the second extraction series did not enhance oleosomes extraction from soybean flour in the second extraction protocol. The results of this study confirm our previous

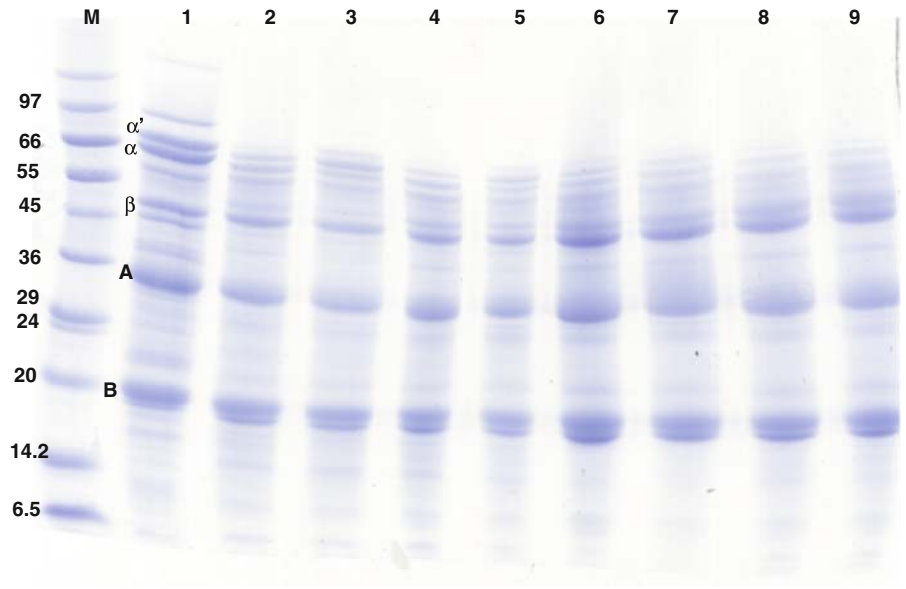

Fig. 4 SDS-PAGE profile of proteins obtained from the aqueous supernatant after two series of reuse: glycinin polypeptides (*A* and *B*) and *β*-conglycinin subunits (*α*′, *α* and *β*) are indicated. Molecular weight marker (*M*), *Lane 1*: starting soybean flour, *Lane 2*: PS (supernatant obtained from an initial extraction, after using soybean flour, buffer and 3% enzymes), *Lane 3*: R1-0% (supernatant obtained after reusing PS with no enzymes), *Lane 4*: R1-3% (supernatant obtained after reusing PS with 3% enzymes), *Lane 5*: R2-0-0% (supernatant obtained after reusing R1-0% with no enzymes), *Lane 6*: R2-0-3% (supernatant obtained after reusing R1-0% with 3% enzymes), *Lane 7*: R2-3-0% (supernatant obtained after reusing R1-3% with no enzymes), *Lane 8*: R2-3-3% (Supernatant obtained after reusing R1-3% with 3% enzymes). 80 μg protein loaded per lane

findings [6]. The oleosome extraction yield is not only influenced by the cellulase/pectinase concentrations, but also by a mechanical disruption of the cell wall obtained after 3 min of cell lysis with a blender.

Protein was the third predominant constituent in the supernatant after two recycling protocols. In the PS, protein concentrations of 4.10 ± 0.35 g/100 mL were obtained (Table 1). This value gradually increased when the supernatant was recycled for oleosome extraction from fresh soybean flour for the two protocols. An increase of protein content in the supernatant after two recycling protocols was not surprising and can be interpreted by high content of protein (39% d.b.) in the starting soybean flour. Other parameters, including the mechanical process to obtain oleosomes and the use of enzyme capable of degrading the cell wall network also increased the extractability of protein via dissolution and/or diffusion kinetics.

Peptide Profile

The distribution of individual proteins in the aqueous supernatant was investigated by SDS-PAGE (Fig. 4) after ultrafiltration of the supernatant. The soybean starting material displayed protein bands eluting at positions equivalent to molecular masses of approximately 80, 75, 50, 34 and 18 kDa (lane 1 and 10). The first three high molecular weight bands correspond to the *α*′, *α* and *β*-subunits of *β*-conglycinin which migrate at higher apparent molecular weights since they are glycopeptides. The two other bands having molecular masses of approximately 34 and 18 kDa correspond to the acidic and basic polypeptides, respectively, of glycinin. These results are consistent with earlier reports on structural characteristics of soybean glycinin and *β*-conglycinin [15–17]. Electrophoretic patterns of the glycinin and *β*-conglycinin fractions were determined in the aqueous supernatants obtained (Fig. 4). The results indicate that even after two protocols of recycling the supernatant with or without adding enzyme, the peptide profiles contained the glycinin fractions were well separated into acidic and basic subunits. This observation can be attributed to the compact tertiary structure of the basic subunits of glycinin, which protects most of the peptide bonds from protease digestion.

The peptide profile obtained when the supernatants were reused without adding enzyme (lane 3 and 5) were similar to a traditional soy protein water extract (lane 1), but with a decrease of intensity on the *α*′ and *α* subunits of *β*-conglycinin. Some differences in the electrophoretic patterns between the recycled supernatant were observed; adding 3% enzymes when reusing the supernatant showed a disappearance of the *α*′ and *α* subunits of the *β*-conglycinin and appearance of new peptides (lanes 4, 6–9). This suggests that protease side activities were present in the enzyme preparations.

Effect of Protease Inhibitor

To further verify the presence of protease side activity in the enzyme preparation, a protease inhibitor cocktail for yeast and fungi (0.5 mL) was added to the soybean–buffer–enzyme mixture before the incubation step. Oleosome isolation was then performed as previously described. The resulted aqueous supernatant was ultrafiltered and the retentate analyzed by SDS-PAGE. Lane 1b of Fig. 5 is the gradient polyacrylamide gel containing the different fractions of proteins after using a protease inhibitor during the fractionation. As expected, no disappearance of the α' and α subunits of the β-conglycinin was observed with the use of 3% enzymes mixture. This result suggests that, under our experimental conditions, Multifect® Pectinase FE, Multifect® GC, Multifect® CX B have a protease contaminant. Using the protease inhibitor in oleosomes extraction with only one Genencor enzyme indicated that the protease activity was present in the Multifect® Pectinase FE and not in Multifect® GC or Multifect® CX B (results not shown). Similar degradation patterns were observed by Jung and others [18] when using higher concentration (5 and 10%) of Multifect pectinase to improve protein extraction yields from defatted soy flakes.

Quantification of Glycinin and β-Conglycinin and Their Subunits by Densitometry

Densitometric analysis was used to quantify the two major storage proteins, glycinin and β-conglycinin and their subunits (acidic and basic subunits for glycinin, α', α and β for β -conglycinin) of starting soybean flour and aqueous supernatants separated on SDS-PAGE (Fig. 4) in triplicate. The amount of glycinin per total protein for the starting soybean flour was 37.57 ± 1.34% (Table 2). These results are comparable with those reported by Hughes and Murphy [19] when studying the varietal influence on the quality of glycinin in soybean. The data in Table 2 indicate that glycinin constitutes between 36.20 and 41.14% of the protein in the aqueous supernatants. There was no difference between the amount of glycinin per total protein of aqueous supernatants and soybean flour. This indicates that neither enzyme treatment and nor the two protocols of recycling the supernatant have an impact on glycinin content recovered in aqueous supernatant.

The β-conglycinin fraction and its subunits (α', α and β) were determined in soybean flour and in aqueous supernatants (Table 2). Soybean flour β-conglycinin content was 30.16 ± 2.01%. The data for glycinin and β-conglycinin

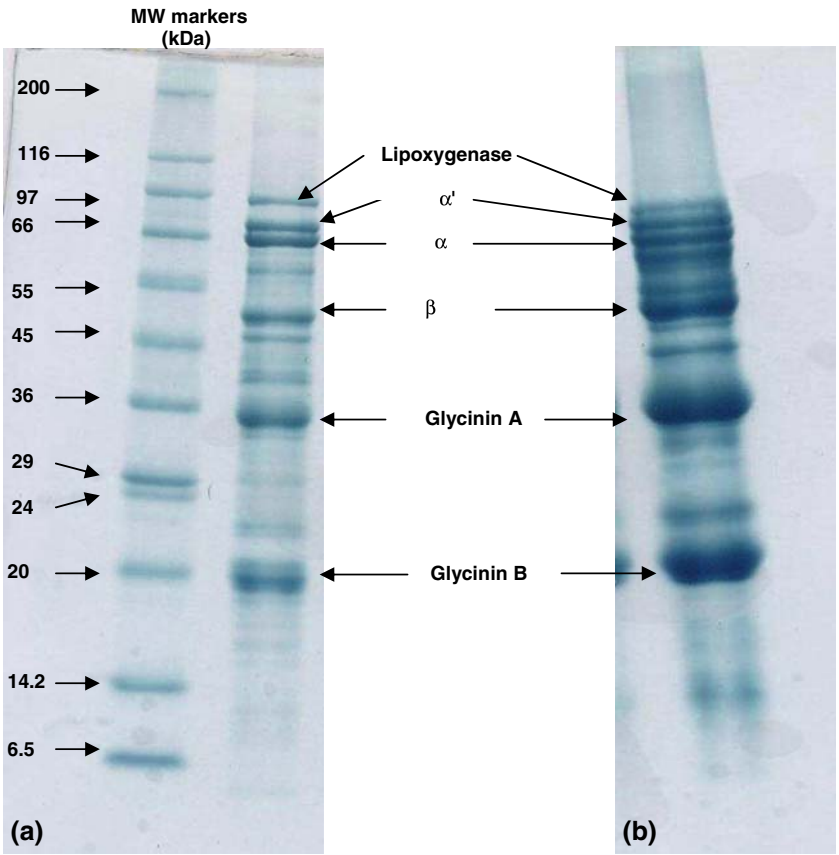

Fig. 5 SDS PAGE profile of proteins obtained from soybean flour with the aqueous supernatant after using protease inhibitor: glycinin polypeptides (*A* and *B*) and β-conglycinin subunits (α', α and β) are indicated. Lane *1a* Starting soybean flour, Lane *1b* supernatant obtained after making an extraction with protease inhibitor. 60–80 µg proteins loaded per lane

Table 2 Distribution of glycinin and β-conglycinin fractions of starting soybean flour and aqueous supernatants

Fractions	Acidic	Basic	Glycinin
Starting soybean flour (% of total protein)			
	14.95 ± 82^{a}	22.63 ± 2.10^{ab}	37.57 ± 1.34^{ab}
Aqueous supernatants (% of total protein)			
PS	15.01 ± 0.73^{a}	21.56 ± 0.44^{ab}	36.57 ± 0.91^{a}
R1-0%	14.42 ± 1.90^{a}	22.66 ± 2.96^{ab}	37.08 ± 3.49^{a}
R1-3%	15.38 ± 1.21^{a}	20.82 ± 3.61^{a}	36.20 ± 3.14^{a}
R2-0-0%	13.20 ± 0.31^{a}	26.14 ± 1.32^{b}	39.94 ± 1.10^{ab}
R2-0-3%	18.39 ± 2.78^{b}	22.35 ± 2.33^{ab}	38.29 ± 3.94^{ab}
R2-3-0%	13.68 ± 1.54^{a}	22.92 ± 2.34^{ab}	36.60 ± 1.55^{a}
R2-3-3%	20.35 ± 1.13^{b}	20.79 ± 0.61^{a}	41.14 ± 1.50^{b}

Fractions	α′	α	β	β-Conglycinin
Starting soybean flour (% of total protein)				
	6.89 ± 0.36^{c}	11.64 ± 0.42^{c}	11.60 ± 1.09^{ab}	30.16 ± 2.01^{c}
Aqueous supernatants (% of total protein)				
PS	ND	2.26 ± 0.41^{a}	9.80 ± 1.06^{ab}	12.05 ± 1.46^{a}
R1-0%	2.31 ± 0.08^{b}	5.74 ± 1.24^{b}	10.28 ± 1.51^{ab}	18.38 ± 2.40^{b}
R1-3%	ND	1.99 ± 0.33^{a}	10.14 ± 1.28^{ab}	12.14 ± 1.21^{a}
R2-0-0%	1.67 ± 0.24^{a}	4.53 ± 0.45^{b}	12.57 ± 2.76^{b}	19.42 ± 2.06^{b}
R2-0-3%	ND	2.50 ± 0.81^{a}	9.87 ± 0.11^{ab}	12.37 ± 1.25^{a}
R2-3-0%	ND	2.47 ± 0.82^{a}	9.35 ± 0.08^{a}	12.29 ± 0.00^{a}
R2-3-3%	ND	ND	9.86 ± 0.84^{ab}	9.86 ± 0.84^{a}

ND non-detectable; means in the column for each fraction with different superscript letters are significantly different at $P < 0.05$

Means in rows with different superscript letters are significantly different at $P < 0.05$

PS supernatant obtained from an initial extraction, after using soybean flour, buffer and 3% of enzymes. *R1-0%* supernatant obtained after reusing PS with no enzymes, *R1-3%* supernatant obtained after reusing PS with 3% enzymes, *R2-0-0%* supernatant obtained after reusing R1-0% with no enzymes, *R2-0-3%* supernatant obtained after reusing R1-0% with 3% enzymes, *R2-3-0%* supernatant obtained after reusing R1-3% with no enzymes, *R2-3-3%* supernatant obtained after reusing R1-3% with 3% enzymes

content reported in this paper are comparable to those reported by others [20] for different soybean varieties, which indicates that glycinin and β-conglycinin constituted between 55 and 75% of the total soybean seed protein. Aqueous supernatants obtained when the extraction was made with 3% enzymes had the lowest amount of β-conglycinin/total protein. The β-conglycinin content of those supernatants ranged from 9.86 to 12.05%. When the aqueous supernatant was recycled with 0% enzyme, the total β-conglycinin contents were higher and ranged from 18.38 to 19.42%. These results showed that the percentage of the β-conglycinin fraction in aqueous supernatants depended upon the type and the amount of enzyme used. The α- and α′-subunits of β- conglycinin were the preferred substrates for protease. On the other hand the proteolysis of the β-subunit of the β-conglycinin was minor. These results indicates that this latter fraction of the β-conglycinin is more resistant to proteolytic attack by proteases than are the α- and α′-fractions, probably due to its chemical and physical structure in which the proteolytic enzymes cannot easily access cleavage sites.

Conclusions

A laboratory-scale study of recycling the aqueous supernatant for oleosome extraction for two protocols with or without adding enzyme resulted in the following conclusions: beside the high percentage of oleosome yield, fractionation allowed the easy extraction of soybean storage protein. The supernatant obtained was rich in minerals and carbohydrates, making the reuse of this media possible. Addition of 3% enzymes increases the oleosome extraction yields in the first recycling protocol, but not significantly in the second recycling protocol. Addition of these enzymes results in the disappearance of the α′ and α subunits of the β-conglycinin. Addition of enzymes continuously in the aqueous supernatant during the recycling is therefore not necessary and will increase the processing cost. Economically feasible production of oleosomes and native protein from soybean full fat flour by recycling the aqueous supernatant is possible. However, to fully exploit the reuse of aqueous supernatant, the feasibility of recycling the aqueous supernatant to isolate oleosomes needs to be demonstrated on a pilot plant scale.

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