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ORIGINAL PAPER

Adsorption of Soy Protein Isolate in Oil-in-Water Emulsions: Difference Between Native and Spray Dried Isolate

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Abstract The purpose of this study was to determine the differences in the emulsifying properties of isolated soy protein prepared in the pilot plant (heated and spray dried) or in the laboratory (unheated and freeze-dried), from the same soy flakes. When the thermal transitions were measured by micro-calorimetry, the protein isolated in the pilot plant showed a very broad thermal transition, while the native isolate showed two distinct transition peaks, attributed to β-conglycinin and glycinin denaturation. Electrophoretic analysis and protein assay of the soluble protein in the fractions revealed a significantly smaller amount of protein recovered in the centrifugal supernatant for the isolated soy protein prepared in the pilot plant than for the native protein. A larger amount of ions was recovered in the pilot plant isolate. However, the thermal treatment of the solutions increased the recovery of the pilot plant isolate proteins in the centrifugal supernatant, with an opposite effect for the native soy protein. A significantly larger amount of pilot plant isolated protein was needed to prepare emulsions with the same characteristics of those prepared with native soy protein. The emulsions prepared with pilot plant isolates showed much lower susceptibility to heating than those prepared with native protein.

Keywords Soy protein isolate · Heat treatment · Denaturation · Emulsion · Emulsifying properties

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Introduction

Soy proteins can be extracted from defatted soybean flakes, and can be purchased in various forms (i.e., soy meal, grits, flour, concentrates and isolates). The advances in processing in the past decade have resulted in a number of soy products with varying functionality and nutritional quality, tailored to different food product applications. Isolated soy proteins (SPI) are commercially available and widely employed as a source of essential amino acids in infant formulas, beverages, nutritional bars, textured food and meat products. However, many studies have shown that challenges remain to be solved to improve the processing functionality of commercial proteins when used in certain applications.

Traditionally, the isolation process of commercial soy proteins starts with defatted soy meal, and the protein is extracted under alkaline conditions. After a step to remove the insoluble residues, the protein solution undergoes isoelectric precipitation. Centrifugation is used to remove precipitated proteins, and after neutralization the isolated protein is heated and spray-dried [1]. Several studies have shown the relationship between the physico-chemical properties of commercial SPI and processing functionality [2–5].

Isolated soy protein is composed mostly of storage proteins, with two globular proteins being the largest component of the final protein isolate, glycinin and β-conglycinin [6]. These two fractions exhibit distinct thermal transitions, with glycinin having a higher denaturation temperature than β-conglycinin. These proteins are heterogeneous complexes containing different subunits. A disulfide linkage is present in between the acidic and basic polypeptides of glycinin, and this protein is present as an hexamer, while β-conglycinin is present as a trimer with

three different subunits α, α' and β, present in various combinations [7, 8]. It has been previously demonstrated that the extent of denaturation has an impact on the processing functionality of the isolated protein, for example, solubility, water holding capacity, viscosity building or gelling [2, 9–11]. In particular, thermal treatment before spray drying can cause denaturation, and may result in changes in the proteins' hydration properties [9].

Generally, proteins will undergo conformational changes upon adsorption at the interface. These changes will depend on their molecular structure; more flexible structures can better adjust to the environmental changes and decrease their free energy during adsorption [12]. Soy proteins have shown the ability to stabilize oil–water or air–water interfaces, by reducing the interfacial tension and form a viscoelastic film at interface [13–15]. A recent study has reported the structural rearrangements of soy proteins at the interface as well as changes with heating on the emulsifying properties of soy proteins [16].

Thermal treatment has a great impact on the structure and functionality of soy proteins [16, 17]. Heat causes structural changes and increased exposure of the hydrophobic patches of the protein with subsequent aggregation. Heating soy protein isolates was shown to induce the interactions between protein subunits [18], and in the case of glycinin, the aggregation preferentially involves basic subunits [19]. Although several studies have been reported on the effect of heat treatment on the emulsifying properties of milk proteins [20, 21] and some of the general principles on the mechanisms related to heat-induced protein–protein interactions may also apply to soy protein emulsion systems, it is yet unclear how to best utilize soy protein ingredients to stabilize soy protein-based emulsions. Many studies have shown the effect of heat treatment on the emulsifying properties of native soy proteins [16, 22–24], yet, it is unclear to what extent commercial isolates differ from heat-treated laboratory-made isolates, as changes in the processing history and composition of the isolate hinder our ability to directly compare samples. The present study compares two isolates prepared from the same batch of defatted flakes. Freeze drying will be employed for laboratory SPI to minimize protein denaturation, while spray drying will be employed as it is commonly applied in isolates prepared for commercial purposes.

Objective of this work was to determine the differences in the emulsifying properties between two protein isolates prepared from the same batch of soy flakes, but processed either in the laboratory ("native") or in the pilot plant (i.e. heat treated and spray-dried). Particular attention was focused on the effect of further heating on the interactions between the proteins and their adsorption at the interface.

Experimental

Materials

Defatted soy flakes (donated by The Solae Company, with a dispersing index of 90) were used to obtain the protein isolates. Isolated soy protein (SPI) was prepared by suspending the soy flakes in 100 mM Tris–HCl buffer at pH 8.0 in 1:10 ratio (w/v). Soluble proteins were separated by centrifugation at 9,000*g* (Beckman Coulter model J2-21, Fullerton, ON, Canada) for 30 min at 10 °C, then adjusted to pH 4.8 with 2 M HCl and refrigerated at 4 °C for 2 h, subsequently centrifuged at 9,000*g* for 30 min at 10 °C (Beckman Coulter, Model J2-21, Fullerton, ON, Canada). The supernatant was discarded and the precipitated protein was collected and washed with 10 mM sodium acetate buffer at pH 4.8 [suspended to 1:8 ratio (w/v)]. The suspension was centrifuged as described above. The slurry was resolubilized in ultrapure water (Barnstead International, E-Pure™ D4641, Iowa, USA), and the final pH was adjusted to 7.5 with 2 M NaOH. The fraction was dialyzed overnight at 4 °C and freeze dried. Protein contents of all fractions were determined using a combustion method (DUMAS) for nitrogen (Leco FP-528 Mississauga, ON, Canada) using Nx6.25 factor The amount of ions (Ca, K, Mg, Na, P, S) was also determined using microwave assisted nitric acid digestion and analysis by ICP-OES (Varian Inc, Palo Alto, USA) by the laboratory services division of the University of Guelph.

A comparable soy isolate was also prepared in the pilot plant facilities of the Solae Company (The Solae Company LLC., MO, USA), using the same batch of defatted soy flakes. The aqueous extraction of the defatted soy flakes followed by mechanical separation was completed using proprietary methods. However, some details are presented below. The resulting soluble fraction was mostly protein and soluble carbohydrate. The pH of the soluble fraction was reduced to the isoelectric point of soy protein ($\sim$pH 4.5). The precipitated protein was water washed and mechanically separated from the soluble carbohydrates, resulting in a protein fraction that is >90% protein on a moisture free basis. The >90% protein stream was diluted with water and pH adjusted with alkali to neutral followed by a pasteurization step. After cooling, the protein stream was spray dried into its final powder form. When necessary, comparisons were also carried out with a dialyzed batch of soy protein isolate. This isolate was resuspended in ultra pure water in 1:50 ratio (w/v), adjusted to pH 7.5 and stirred overnight at 4 °C. After dialysis for 18 h at 4 °C, the fraction was freeze-dried and stored at −20 °C. Protein analysis as well as ionic compositions was analyzed as described above.

Protein Solubility

To better characterize the solubility of the protein in solution before and after heating (see below), the isolated soy protein (1%, w/v) was resuspended in 50 mM sodium phosphate buffer, pH 7.4 and stirred for 1 h at room temperature then stirred overnight at 4 °C to fully hydrate. The suspension (1 mL) was equilibrated at room temperature and then transferred to an eppendorf tube and centrifuged at 10,000*g* (Brinkmann Instruments, Westbury, NY) for 15 min to separate the insoluble residue. The amount of protein remaining in solution after centrifugation was defined as the amount of soluble protein. The supernatant was removed and extracting buffer was added to the remaining pellet for further analysis using SDS-PAGE. The total protein dispersion and the soluble fraction (i.e. the centrifugal supernatant) were analyzed using DC™ Protein assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with bovine serum albumin as a standard. Solubility was calculated as the amount of protein remaining in the supernatant per initial protein content. Proteins present in the soluble and insoluble phases in soy protein dispersion were also analyzed by SDS-PAGE, as described below.

Emulsion Preparation

The dried soy protein isolate was suspended in 50 mM sodium phosphate, pH 7.4 at various concentrations, ranging from 1.0 to 2.5% (w/v) and stored at 4 °C overnight to fully hydrate. The suspension was pre-homogenized with 10% soy bean oil (w/w) (Sigma-Aldrich, St. Louis, MO, USA) using a dispersing unit (Power Gen 125, Fisher Scientific, Ottawa, ON) for 1 min then immediately homogenized through a Microfluidizer (110S model, Newton, MA) for five passes at an overall input pressure of 300 kPa. The emulsions were then immediately analyzed and then stored at 4 °C for 1 week to determine possible changes in particle size distribution with time.

Heat Treatment

Heating was carried out at two different temperatures, 75 and 95 °C for 15 min using circulating water bath (Thermo Haake, DC10 model, Sigma-Aldrich, St. Louis, MO). These two temperature/time regimes were chosen as they cause selective denaturation of β-conglycinin (75 °C) or both β-conglycinin and glycinin (95 °C) as shown by calorimetric analysis [24]. The solubility of heated protein dispersions was analyzed as described above. Emulsions were prepared either with heated or unheated protein dispersions. When the protein dispersions were heated prior to emulsion preparation, samples were placed into sterile polypropylene tubes and placed in a water bath at the desired heating temperature. Samples were then removed and cooled in an ice bath. Further analyses or emulsification were carried out immediately after the treatment. When emulsions were heat-treated, the oil fraction was homogenized with unheated protein dispersions, and then immediately placed in a water bath, heated at the appropriate conditions and then cooled in an ice bath.

Particle Size Distribution

Static light scattering (Mastersizer 2000S, Malvern Instrument Inc., Westborough, MA) was employed to measure the average mean particle size and particle size distribution as a function of protein concentration under various conditions (i.e., unheated, heated at 75 and 95 °C for 15 min before or after emulsification). A refractive index of 1.47 was used for dispersion (soybean oil) and 1.33 for the continuous phase (ultrapure water). The analyses were conducted with freshly made emulsions and after storage at 4 °C to assess their stability, and the volume-weighted mean particle size ($D_{4,3}$) was reported. In the case of aggregated samples, average particle size distribution data were less reproducible than those determined in the stable system, and the differences from the more stable distributions were taken only as an indication of destabilization, as the calculation of size distribution from the raw scattering data is carried out under the assumption of uniform spherical particles [25].

Differential Scanning Calorimetry

The thermal denaturation behavior of the soy proteins was determined using a VP-DSC microcalorimeter (Microcal Incorporated, North Hampton, MA). Protein samples were diluted to 5 mg/ml with 50 mM sodium phosphate buffer, pH 7.4. Solutions were held at 20 °C for 15 min prior to heating from 20–115 °C with a 1 °C per min scan rate, and then cooled to 20 °C. The instrument contained two measuring cells, a sample and a reference cell. Sodium phosphate buffer was employed as the reference when measuring protein solutions. To determine thermal transition of the protein present at the interface, the emulsions were centrifuged at 10,000*g* (Optima LE-80 K, Beckman Coulter, Brea, CA) at 25 °C for 45 min. The cream layer was then carefully removed from the top layer and dried on filter paper (Whatman no. 1, Fisher Scientific, Ottawa, ON, Canada), and rinsed with ultra pure water, then resuspended in 50 mM sodium phosphate buffer pH 7.4, to the initial volume fraction of oil prior to the run with the same condition described above. A small molecular weight emulsifier (Tween 80, 0.5%) was used as a reference when measuring soy protein-stabilized emulsions. Enthalpy (ΔH)

and denaturation temperature midpoint (T_d) were analyzed using the Origin™ software version 7.0.

SDS-PAGE Electrophoresis

The total protein in solution and the soluble protein were use to determine any differences in the composition of subunits after centrifugation and heat treatment using gel electrophoresis. The loading was not adjusted for protein, to better identify differences between the original solution and the serum phase. Traces of insoluble protein present in the pellet after centrifugation were dissolved in electrophoresis buffer to determine qualitatively if any polypeptides selectively precipitated after heating. The analyses were carried out both under reducing and non-reducing conditions to determine whether disulfide linkages were involved in the stabilization of the aggregates. To separate the soluble protein remaining in supernatant from the precipitant (pellet), 1 mL of protein dispersion [1%, (w/v)] was centrifuged at 10,000*g* (Eppendorf, Brinkmann Instruments, Westbury, NY) at 25 °C for 15 min. Supernatant was removed and the remained precipitant was then used for further analysis. Aliquots (200 μL) of sample (dispersions, soluble fractions, or traces of pellet in the eppendorf tubes) were treated with 210 μL of extraction buffer, containing 50 mM Tris–HCl, 5 M urea, 1% SDS, 4% 2-mercaptoethanol, pH 8.0. The samples were equilibrate at room temperature for an hour then diluted with 210 μL of electrophoresis buffer containing of 125 mM Tris–HCl, 5 M Urea, 1% SDS, 20% Glycerol, 4% 2-mercaptoethanol, pH 6.8. In electrophoretic analysis in non reducing condition, 2-mercaptoethanol was replaced by water in the extraction and the electrophoresis buffer. The samples were then heated at 95 °C for 5 min. After cooling to room temperature, the samples containing fat were centrifuged (Eppendorf, Brinkmann Instruments, Westbury, NY) at 10,000*g* for 10 min, to separate the oil phase before loading the aqueous extract.

Aliquots (5 μL) of all protein samples were loaded onto 12.5% polyacrylamide gel with 4% stacking gel in Bio-Rad mini-protein electrophoresis (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) for proteins separation. Gels were fixed and stained using Bio-Rad Coomassie blue R-250 stain solution (45% methanol, 10% acetic acid and 0.10% Blue R-250) followed by de-staining using 45% methanol, 45% ultrapure water and 10% acetic acid solution then gel was scanned using a SHARP JX-330 scanner (Amersham Biosciences, Baie d'Urfe, Quebec, Canada).

Statistical Analysis

All experiments were performed in triplicate (i.e. three separate experiments) and averages are reported. Statistical significance was tested by comparing standard deviations using the *t* test or ANOVA with a $P < 0.05$.

Results and Discussion

Physicochemical Properties of Proteins

Table 1 summarizes the protein and ionic composition of the various isolates. The laboratory made, "native" soy protein (N-SPI) contained a larger amount of protein than the isolate prepared in the pilot plant (PP-SPI). In addition, the native protein contained a smaller amount of ions than the PP-SPI. The discrepancies of amount of proteins and salt content between the isolates may influence their functionality. It is important to note that it has been previously demonstrated that soy proteins show better solubility at high ionic strength [9, 22]. However, it has also been shown that ions can modify protein adsorption behavior and have adverse effects on the stability of protein-stabilized emulsions [25, 26]. After dialysis, the PP-SPI did not show a significant reduction in the amount of ions present, apart from a decrease in sodium content (a decrease of approx. 20%). These results suggest that the neutralization and the heat-induced denaturation process during pilot plant processing caused an extensive association of ions with the proteins.

Thermal Transition of the Isolates

Micro-differential scanning calorimetry was employed to determine the extent of denaturation of the N-SPI and PP-SPI. The differences in their thermal transitions confirmed that the processing history affected the denaturation state of the proteins in the two isolates. The thermal transition of the N-SPI showed two distinct transition peaks, at 68 and 85 °C, corresponding to β-conglycinin and glycinin thermal denaturaturation, respectively (Fig. 1). These finding are in agreement with previous studies [10, 16, 27]. The thermal transition of the PP-SPI was quite different from that of the N-SPI solution (Fig. 1). The PP-SPI exhibited a single broad peak starting at much earlier temperature, about 50 °C, with a peak maximum at 79 °C. This thermal transition indicated that rearrangement of the protein structures occurred, with the formation of protein complexes with lower thermal stability. This behavior has never been reported before; however, it has been previously shown that commercial isolates may show different degrees of denaturation, with consequences to the functional properties of the protein [2, 5]. Dialyzed PP-SPI was also tested and no significant changes were observed in the thermal transition of the isolate compared to the same non-dialyzed proteins (data not shown). The results shown in

Table 1 Protein and ionic composition measured for the native (N-SPI) and pilot plant isolates (PP-SPI), dialyzed (D) and non dialyzed (ND)

Sample	Protein content (%)	Ions (μg/g)					
		Ca	Mg	P	Na	K	S
N-SPI	97 ± 2	280 ± 14	230 ± 14	3,950 ± 495	9,400 ± 849	470 ± 99	6,300 ± 212
PP-SPI (ND)	86 ± 1	1,700 ± 70	510 ± 14	8,100 ± 919	13,000 ± 1,414	770 ± 57	5,700 ± 141
PP-SPI (D)	86 ± 1	1,500 ± 1	465 ± 7	7,450 ± 212	9,450 ± 212	570 ± 14	5,800 ± 141

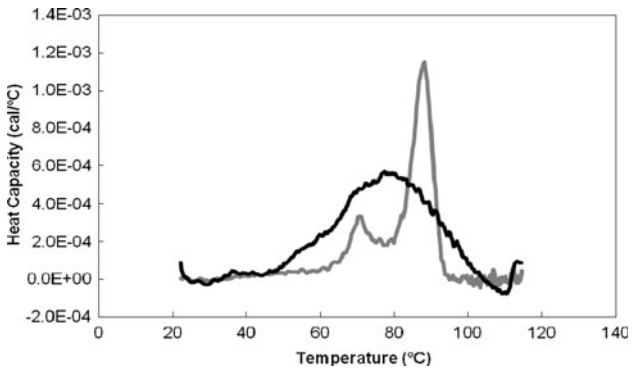

Fig. 1 Endothermic transitions as measured by micro-DSC for 1% soy protein dispersed in 50 mM sodium phosphate, pH 7.4. N-SPI (*gray line*); PP-SPI (*black line*). The thermograms are representative of three replicate experiments

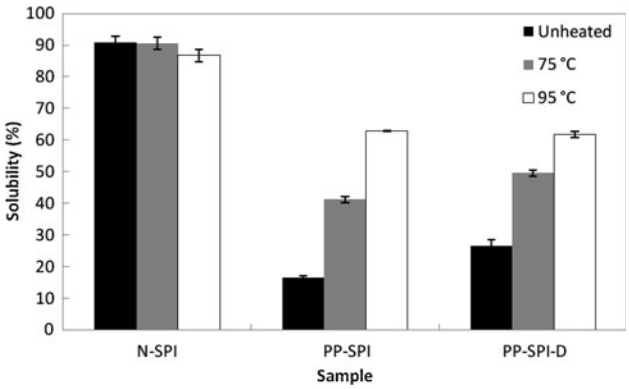

Fig. 2 Effect of heat treatment on the solubility of native soy protein isolate (N-SPI), pilot plant soy protein isolate (PP-SPI), dialyzed pilot plant soy protein isolate (PP-SPI-D). Protein suspensions were heated at 75 or 95 °C for 15 min. Values are the average of three replicate experiments

Fig. 1 confirm that the processing of the protein during the pilot plant isolation (most likely heat treatment and spray drying) caused changes in the supramolecular structure of the soy proteins. It is important to note that the soy proteins prepared in the pilot plant still showed quaternary structure changes during heating.

The Effect of Heating on Protein Solubility

To evaluate differences in protein solubility and how it may be affected by thermal treatment, centrifugal supernatants were analyzed for protein content. In addition, SDS-PAGE electrophoresis was also carried out, to determine possible differences in the distribution of the subunits in the supernatant with heating. When present, the pellet was also analyzed to determine possible changes in the polypeptide composition. Figure 2 illustrates the effect of thermal treatment on the solubility of the various isolates. Approximately 91% of N-SPI was soluble, while only 17% of PP-SPI remained in soluble phase in unheated samples. These findings were not surprising, considering the reports on the effect of thermal denaturation on protein solubility [28]. The removal of ions from the PP-SPI increased the solubility of the unheated sample, but still less than 40% protein was recovered in the supernatant after dialysis.

Electrophoresis performed under reducing and non-reducing conditions (Fig. 3) also showed full agreement with the solubility data. The majority of the polypeptides in the unheated N-SPI solutions were found in the centrifugal supernatant, most likely present as soluble aggregates. On the other hand, in the unheated PP-SPI, a significant portion of the proteins was recovered in the precipitate fraction (Fig. 3b, d). These results, together with the micro-DSC results shown in Fig. 1, suggest that the isolation process carried out in the laboratory was mild enough to maintain the proteins in their native structure, as previously suggested [10]. Alternatively, the industrial isolation processes always require application of heat to inactivate enzymes and anti-nutritional components, and this will lead to protein denaturation and loss of protein solubility. The loss of solubility is caused by the polymerization of disulfide bonds between glycinin and β-conglycinin subunits and the aggregation and molecular rearrangements from the exposure of hydrophobic patches [18].

Heating affected the solubility of the isolates to different degrees (Figs. 2, 3). In N-SPI, heating caused a slight decrease in solubility from 91 to 87% after heating at 95 °C for 15 min. Most subunits seemed to be present in the pellet. In addition, Fig. 3c showed a band was present in the N-SPI before heating (Band 1, migrating at about 31 kDa) and after heating at 75 °C, while it was no longer present in the N-SPI after heating at 95 °C. Heating of the PP-SPI caused an improved solubility of the protein dispersions, as significantly more protein was recovered in the

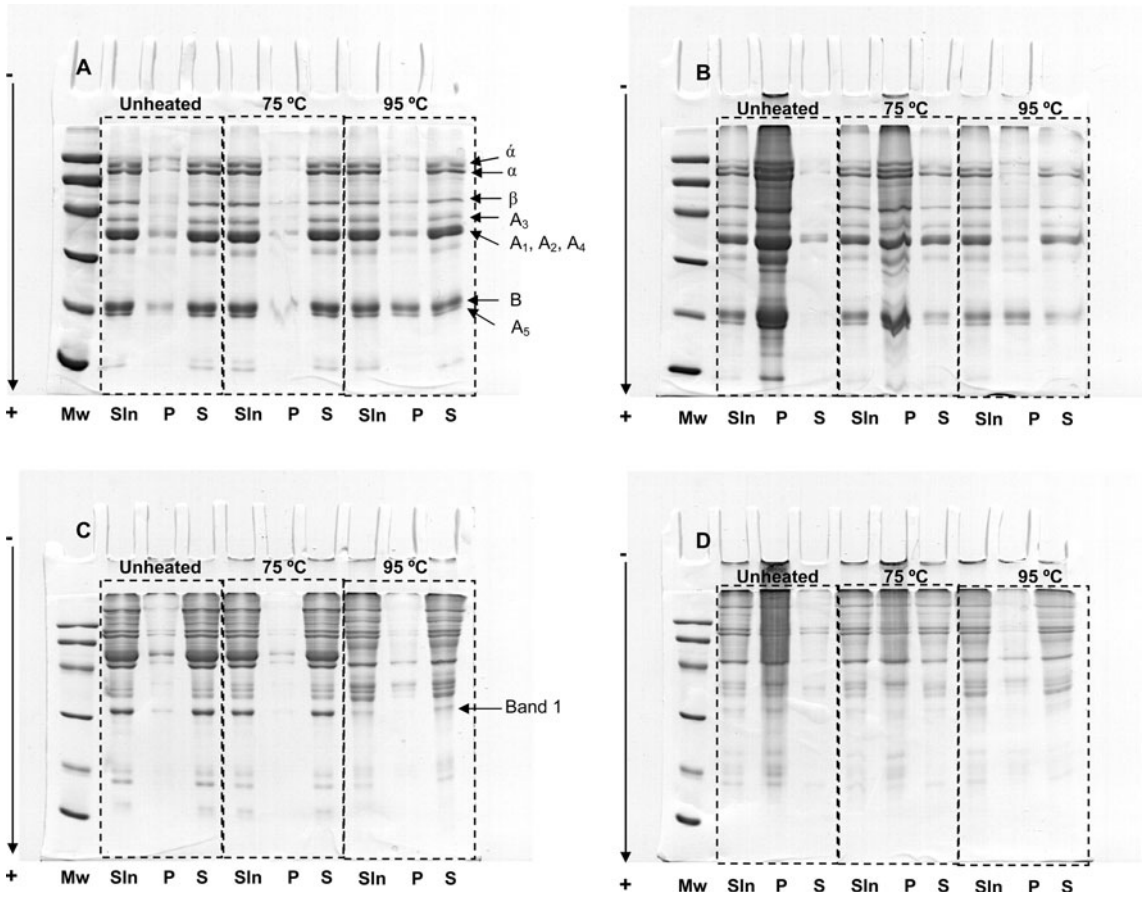

Fig. 3 SDS-PAGE analysis carried out under reducing conditions (a, b) and non reducing conditions (c, d) of 1% SPI (a, c) and 1% PP-SPI (b, d). Solutions (Sln); (P) precipitate and (S) soluble phase after centrifugation. (MW) molecular weight marker: phosphorylase B, 97,400 Da; BSA, 66,200 Da; ovalbumin, 45,000 Da; carbonic anhydrase, 31,000 Da; soybean trypsin 21,500 Da; lysozyme 14,400 Da. Subunits are identified. Proteins samples were unheated, or heated at 75 or 95 °C for 15 min

soluble fraction after heating at 75 and 95 °C (Fig. 2). The dialysis of the PP-SPI did not seem to cause differences in the solubility of the PP-SPI. The electrophoretic profile of the PP-SPI was also quite different from that of the N-SPI. Less bands were present in the supernatant, and less in the low molecular weight region (compared to the N-SPI) (Fig. 3b, d), suggesting the absence of basic polypeptides in the supernatant. Interestingly, the polypeptide pattern under non-reducing conditions of the PP-SPI was similar to that of the N-SPI after heating at 95 °C for 15 min.

Emulsifying Properties

Several studies have demonstrated that protein denaturation affects the functional properties of soy protein isolates [2, 9–11]. It has also been reported that protein solubility is often correlated with emulsifying properties [29]. The solubility results clearly showed a different effect of heating on protein solubility in N-SPI compared to PP-SPI. As heating improved the solubility of PP-SPI, it may be possible to hypothesize that heating may also improve the emulsifying properties of the PP-SPI.

To determine the emulsifying properties of N-SPI and PP-SPI, emulsions were prepared with different concentrations of proteins and their particle size distribution was measured using static light scattering. The average size of the emulsion droplets as a function of concentration may suggest differences in the emulsifying activities between the isolates, while a change in size distribution over storage time may assist in evaluating the stability of the emulsions. The oil droplet size decreased with protein concentration, and N-SPI clearly was more effective than PP-SPI in creating small droplet sizes, more evidently at the low concentrations (Table 2). This difference could not be explained simply by the difference in protein concentration between the two isolates (Table 1). At concentrations >2% there was no significant difference in the average size of the emulsions between the two treatments. A minimum of 2.5% SPI was needed for the PP-SPI to achieve a monomodal distribution of sizes comparable to that of emulsions

Table 2 Average mean droplet diameter *D* [4, 3] measured using integrated light scattering for 10% soy oil emulsions prepared with N-SPI and PP-SPI at various concentrations

Sample	N-SPI		PP-SPI	
	Freshly made	After 1 week	Freshly made	After 1 week
Unheated				
1.5% SPI	0.58 ± 0.1	2.99 ± 1.8	3.73 ± 1.4	3.71 ± 0.7
2.0% SPI	0.45 ± 0.1	0.81 ± 0.4	0.88 ± 0.2	2.21 ± 1.1
2.5% SPI	0.44 ± 0.1	0.90 ± 0.9	0.44 ± 0.1	0.70 ± 0.6
Emulsion prepared with heated solution				
1.5% SPI	8.72 ± 1.5	8.92 ± 0.9	6.57 ± 1.7	9.33 ± 0.3
2.0% SPI	1.98 ± 0.1	2.71 ± 1.4	0.83 ± 0.1	1.17 ± 0.4
2.5% SPI	N/a	N/a	0.50 ± 0.1	0.75 ± 0.2
Heated emulsion				
1.5% SPI	0.88 ± 0.5	0.96 ± 0.2	4.42 ± 1.1	5.21 ± 1.9
2.0% SPI	0.43 ± 0.1	0.47 ± 0.1	0.80 ± 0.2	0.9 ± 0.1
2.5% SPI	0.43 ± 0.1	0.44 ± 0.1	0.37 ± 0.1	0.41 ± 0.1

Unheated emulsions are compared with emulsions prepared with a solution heated at 95 °C or emulsions heated at 95 °C after homogenization. Values are the averages of three independent experiments

prepared with N-SPI, and with a similar average size after 1 week of storage (see Table 2).

The aggregated state of PP-SPI and the large amount of ions present influenced the emulsifying properties of the isolate [26]. However, it is important to note that because of the heating treatment conducted on the PP-SPI before drying, the ions were mostly associated with the protein, and dialysis did not substantially lower the ion concentration. On the other hand, as shown in Fig. 4, the size distribution of the emulsions prepared with PP-SPI dialyzed was different than that of emulsions prepared with PP-SPI. The differences were also confirmed using light microscopy (data not shown). The free ions promoted aggregation of the oil droplets (Table 3), as after dialysis it was possible to prepare emulsions with a smaller average droplet diameter. The destabilization of the oil droplets induced by the presence of ions has been recently reviewed [22], and it is possible to hypothesize that in the case of soy protein stabilized emulsions, the destabilization occurred because of a decrease in the electrostatic repulsion of the droplets, a reduced net charge of the proteins per se, or bridging between the droplets by the protein aggregates. The findings were in full agreement with previous studies on salt destabilization of soy emulsions [13, 23]. Figure 5 illustrates the differences in the size distribution of the emulsion droplets with storage time. Although emulsions prepared with N-SPI showed a monomodal distribution of sizes even when prepared with 1% SPI, the emulsions showed some aggregation after 1 week of storage (Fig. 5a, b). It has been previously demonstrated that soy proteins still hold a quaternary

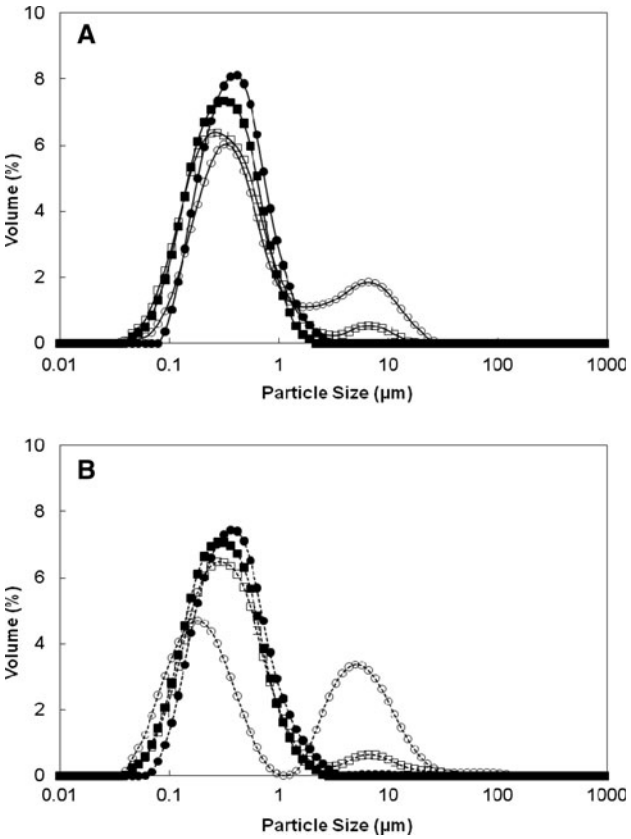

Fig. 4 Particle size distribution measured by integrated light scattering for 10% oil in water emulsions prepared with Non dialyzed and dialyzed PP-SPI at different concentrations. Non-dialyzed PP-SPI at 1.5% (*open circles*), 2.0% (*open squares*) and Dialyzed PP-SPI at 1.5% (*closed circles*) and 2.0% (*closed squares*). Fresh emulsions (a) and the same emulsions after 1 week of storage at 4 °C (b)

Table 3 Average mean droplet diameter *D* [4, 3] measured using integrated light scattering for 10% soy oil emulsions prepared with non-dialyzed and dialyzed PP-SPI at various concentrations

Sample	Non-dialyzed		Dialyzed	
	Freshly made	After 1 week	Freshly made	After 1 week
Unheated				
1.5% SPI	3.7 ± 1.4	3.7 ± 0.7	0.6 ± 0.1	0.6 ± 0.1
2.0% SPI	0.9 ± 0.2	2.2 ± 1.1	0.4 ± 0.01	0.5 ± 0.1
Emulsion prepared with heated solution				
1.5% SPI	6.6 ± 1.7	9.3 ± 0.3	1.9 ± 0.4	2.0 ± 0.1
2.0% SPI	0.8 ± 0.1	1.2 ± 0.4	0.5 ± 0.1	0.6 ± 0.1
Heated emulsion				
1.5% SPI	4.4 ± 1.1	5.2 ± 1.9	1.5 ± 0.3	2.2 ± 0.4
2.0% SPI	0.8 ± 0.2	0.9 ± 0.1	0.4 ± 0.03	0.7 ± 0.2

Unheated emulsions are compared with emulsions prepared with a solution heated at 95 °C or emulsions heated at 95 °C after homogenization. Values are average of three independent experiments

structure when adsorbed at the interface [16]. Therefore, it is possible to hypothesize that during storage, the adsorbed proteins may undergo some rearrangements at the interface and interact with one another, leading to droplet aggregation. In the case of emulsions prepared with PP-SPI, although more protein was needed to obtain a monomodal distribution of small oil droplets, the particle size distributions did not change over 1 week of storage (Fig. 5c, d).

To determine the effect of heating treatment on the emulsifying properties of the proteins, the SPI dispersions were heated at 95 °C prior to homogenization and the droplet size of emulsion was examined. In N-SPI, heating the solutions caused a dramatic increase in the average droplet size, to values larger than those of emulsions prepared with unheated PP-SPI (Table 2). These results clearly indicated that the difference in the supramolecular structure between the N-SPI and PP-SPI affected the emulsifying properties of the proteins. Indeed, after heating N-SPI, aggregates form [16] and these aggregates cover the emulsion droplets. Moreover, it was demonstrated that the effect of heat-induced the interaction between adsorbed and unadsorbed proteins resulted in extensive bridging flocculation of emulsion droplets [16]. It is clear that the differences in the supramolecular structures formed during heating affect the emulsifying properties of the SPI: the average sizes of emulsions with PP-SPI were smaller than those obtained with heated N-SPI solutions.

The average particle size of the emulsions prepared with the heated solutions was also compared to that of emulsions prepared with the PP-SPI and then heated under the same conditions. The results are summarized in Table 2 as a function of protein concentration. Within the same concentration, heating before or after homogenization for the PP-SPI did not show significant differences in the average size of the fresh emulsions. Heating the emulsion containing 2.5% protein after homogenization did not cause changes in the average droplet size after 1 week of storage. Interestingly, heating after homogenization of PP-SPI emulsions resulted in a small average particle size, with very little change after 1 week of storage at 4 °C, similarly to what observed for N-SPI. It was concluded that heating does not affect the emulsifying stability of the PP-SPI fraction. This was not the case for N-SPI solutions heated before homogenization (Table 2). When aggregates are present in solution, more protein will be required to obtain full coverage of the interface. These results confirm what previously reported on the emulsifying behavior of N-SPI [16]: heating the solution before homogenization increased the surface load of N-SPI emulsions.

Protein Composition at the Interface

The type of protein adsorbed at the oil/water interface was determined using SDS-PAGE, to identify whether any

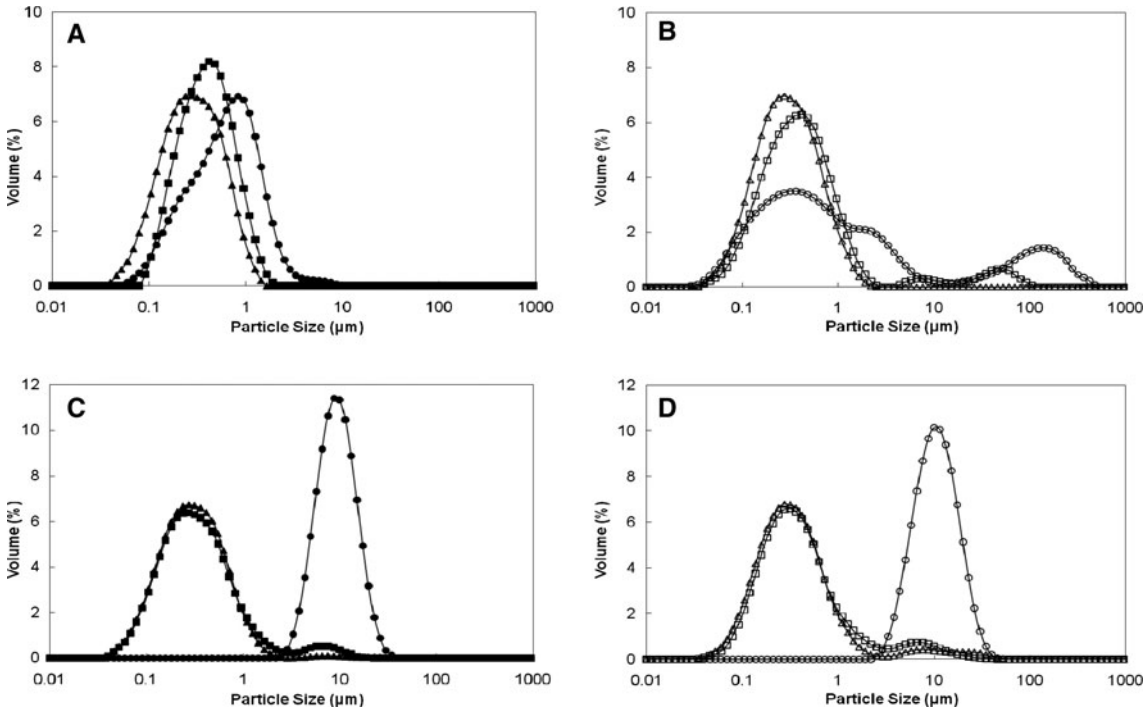

Fig. 5 Particle size distribution measured by integrated light scattering for 10% oil in water emulsions prepared with N-SPI (a, b) and PP-SPI (c, d) at different concentrations. (*closed circles*, *open circles*) 1% SPI; (*closed squares*, *open squares*) 2.0% SPI; (*closed triangles*, *open triangles*) 2.5% SPI; Fresh emulsions (a, c) and the same emulsions after 1 week of storage at 4 °C (b, d)

preferential subunits absorbed at interfacial layer depending on the type of SPI employed. This analysis was carried out after centrifugation of the emulsions and separation of the cream phase. In both N-SPI and PP-SPI emulsions, all the subunits were recovered in both serum and cream phases (data not shown). The main difference between the two emulsions was the presence of a significant amount of protein in the insoluble fraction in the PP-SPI emulsions, confirming the lower solubility of the PP-SPI dispersion, compared to the N-SPI.

State of Adsorbed Proteins at the Interface

To better understand the state of the protein at the oil–water interface, the thermal transitions of the proteins once adsorbed at the interface were measured using micro-calorimetry. Figure 6 compares the thermal transitions of the protein adsorbed at the interface (where the cream phase was separated from the unadsorbed fraction) with those recorded from the original solution. Previous studies have reported that when proteins adsorb at the interface, they adopt conformations that minimize their free energy. This may cause a decrease in the thermal transition if surface denaturation occurs [30]. The thermal transition of the N-SPI at the interface (Fig. 6a) still showed two distinct transition peaks for β-conglycinin and glycinin, with the appearance of a third peak at about 90 °C, with a maximum at 95 °C. This latter peak has been attributed before to a rearrangement of β-conglycinin fraction at the interface [27]. These results clearly showed that the proteins adsorbed at the interface underwent structural rearrangements, and that they maintained their supramolecular structure. The transition at high temperature suggests an increase in the stability of the adsorbed complex formed at the interface.

On the other hand, the cream phase of emulsions prepared with the PP-SPI showed a much different transition compared to that of the protein in solution (Fig. 6b). The protein in solution, as already shown in Fig. 1, showed a broad transition starting at low temperature (about 50 °C). The protein present in the cream phase of PP-SPI emulsions showed much lower enthalpies of transition, and two major peaks, with a maximum at 70 and 95 °C. The largest transition peak was at temperatures >90 °C. These results may suggest that most of the protein present at the interface of PP-SPI emulsions are either more surface denatured, or forming a supramolecular complex with a higher temperature of denaturation, as already discussed for N-SPI samples.

Figure 6 also compares the thermal transitions between cream separated from unheated emulsions or emulsions heated. While in the case of N-SPI most thermal transitions disappeared, in PP-SPI the peak at high temperature (with a maximum at 95 °C) was unchanged, once again supporting the previous results of a higher thermal stability of the PP-SPI proteins compared to N-SPI.

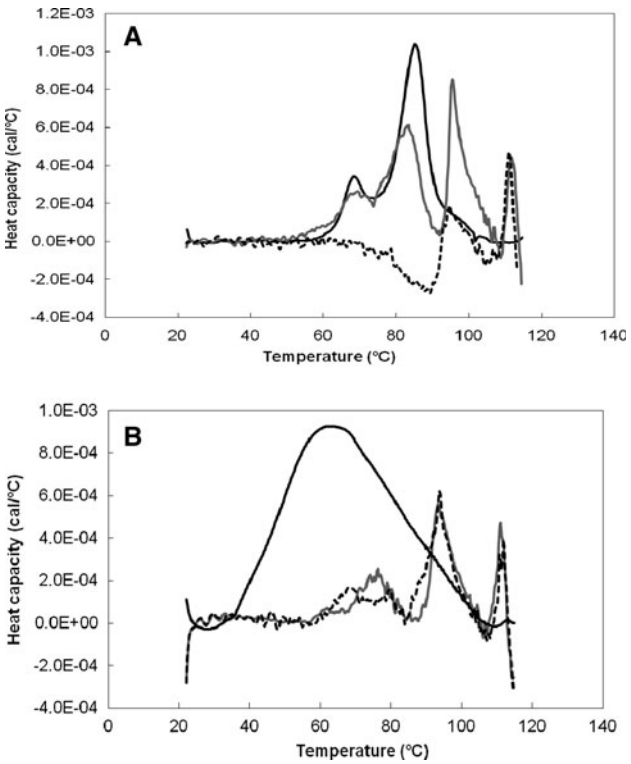

Fig. 6 Thermal transitions of 2% N-SPI (a) and 2% PP-SPI (b) in solution (*solid black line*), and adsorbed protein at the interface (cream phase separated from emulsion) (*solid gray line*) or adsorbed protein at the interface (cream phase separated from the emulsion) after the emulsions were heated at 95 °C for 15 min (*dashed line*)

Conclusions

In conclusion, this study showed that the processing history results in very significant differences in the emulsifying behavior of the isolates. The compositional differences can explain only in part the differences between these proteins. Heating is a necessary step in the preparation of SPI, and this research clearly showed that the formation of heat-induced supramolecular aggregates affect the emulsifying properties of the soy protein, however, does also impart further stability to the emulsion during storage.

Although a larger amount of protein is necessary to achieve complete coverage of the oil water interface in PP-SPI compared to N-SPI, PP-SPI emulsion droplets are stable with storage. The reason behind the lower emulsifying capacity of the PP-SPI compared to N-SPI is most likely the higher extent of aggregation present in the PP-SPI as well as the larger amount of ions present in the isolate. It is important to note that heating improved the

solubility of the PP-SPI, while slightly decreased the solubility of N-SPI. Micro-calorimetry measurements of the protein adsorbed on the oil droplets suggested that supramolecular aggregates were present at the interface of PP-SPI emulsions, as only transition peaks >85 °C were noted in the cream phase, in spite of the wide thermal transition peak shown in the PP-SPI in solution.

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