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Effect of isolation conditions on structural properties and surface behavior of soy-whey proteins

Romina Ingrassia[1], Pablo Antonio Sobral[2], Patricia Hilda Risso[1], Gonzalo Gastón Palazolo[3] and Jorge Ricardo Wagner[3]*

[1]Instituto de Física Rosario (IFIR), Universidad Nacional de Rosario (UNR), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Facultad de Ciencias Veterinarias, 2000 Rosario, Santa Fe, Argentina.
[2]Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), B1900AJJ La Plata, Buenos Aires, Argentina.
[3]Universidad Nacional de Quilmes (UNQ), CONICET, Laboratorio de Investigación en Funcionalidad y Tecnología de Alimentos (LIFTA), Departamento de Ciencia y Tecnología, B1876BXDBernal, Buenos Aires, Argentina.

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ABSTRACT

In this study, the impact of isolation conditions on structural and surface properties at the air/water interface of soy-whey proteins (SWP) was assessed. SWP were obtained by precipitation of soy-whey (at pH 4.5 or 8.0) with acetone or ammonium sulfate. Despite the fact that all SWP samples exhibited similar electrophoretic patterns, they showed different protein content (from 54.2 to 98.2% w/w). When precipitation was performed at pH 4.5, SWP samples evidenced a decrease of protein solubility (S_P) and thermal stability, while the precipitation with acetone promoted the enrichment in polysaccharides and minerals. For all samples, intrinsic fluorescence, surface hydrophobicity and Fourier transform infrared (FTIR) studies revealed structural changes correlated to protein unfolding and aggregation processes. However, the surface behavior can be predicted from these studies mainly due to differences in surface hydrophobicity and the differential contribution of insoluble aggregates. The heating of SWP samples enhanced the surface activity, regardless of the pH of the raw material and the isolation method. These results can be useful as a reference research and as a starting point for industrial exploitation of proteins from soy wastewater.

INTRODUCTION

Soy proteins, mainly as protein isolates, due to their structural characteristics and wide range of functionalities, have been well-established in food industry and they also show promissory future in other industrial applications (Hettiarachchy and Kalapathy, 1998; Nishinari et al., 2014; Fahmy et al., 2010, 2014). During the manufacture of soy protein isolates, soy-whey is generated as residual liquid. In order to avoid water pollution, this effluent should be conveniently treated in a sewage plant before being discharged, increasing in this way the cost for production (Belén et al., 2012). However, because of the fact that soy-whey contain valuable proteins and other exploitable compounds, such as isoflavones and oligosaccharides, the recovery of such components will be profitable for plants producing soy protein isolates (Li et al., 2014; Rickert et al, 2004).

Soy-whey proteins are mainly constituted by trypsin inhibitors (Kunitz and Bowman-Birk trypsin inhibitor, KTI and BBTI, respectively), lectin, β-amylase and lipoxygenase (Sorgentini and Wagner, 1999; Thrane et al., 2017). Despite its known antinutritional effect, these biologically active proteins have a high sulfur amino acid content and are soluble in wide ranges of pH and ionic

*Corresponding author. E-mail: jorge.wagner@unq.edu.ar. Tel: +54 11 43657100 (ext. 5615). Fax: +54 11 43657132.

strength (Tan-Wilson and Wilson, 1986). However, soy-whey proteins can be inactivated by mild hydrothermal treatments (Lajolo and Genovese, 2002; Vagadia et al., 2017). On the other hand, previous works have also reported about their emulsifying (Palazolo et al., 2004; Ray and Rousseau, 2013; Sorgentini and Wagner, 2002), foaming (Sorgentini and Wagner, 2002) and biological properties (Lassissi et al., 2014).

Although in previous works soy-whey proteins were obtained from soy-whey by salting out with ammonium sulfate and cold acetone precipitation (Sobral and Wagner, 2007, 2009; Sobral et al., 2010), little is still known about the effects of these preparation methods on the structural and functional properties of these protein samples.

The aim of this paper was to study the impact of preparation methods of soy-whey proteins on their structural properties and surface behavior at the air/water interface. Because of the fact that the structure and functionality of proteins are highly influenced by environmental conditions, the effects of precipitation method, pH variation during the precipitation step and thermal treatment were especially assessed.

MATERIALS AND METHODS

Materials

Defatted soy flour, provided by Solae (Barueri, São Paulo, Brazil), contained 95 g dry solids per 100 g flour and its composition (% w/w in dry basis, as given by the provider) was: crude protein (N×6.25), 56.0; ash, 7.0; total lipids, 3.5; total dietary fiber, 14.0. D-glucose and 1-anilino-8-naftalen-sulfonate ammonium salt (ANS) were purchased from Sigma-Aldrich (MO, USA). Acetone (> 99.5%) was purchased from J. T. Baker (Avantor, PA, USA). Low molecular weight marker kit was purchased from GE Healthcare (GE Corp., USA). Distilled water was always used in all assays and all the other chemicals were analytical grade reagents.

Preparation of soy-whey protein samples

To prepare samples from soy-whey, firstly, the aqueous solubilization of defatted soy flour at pH 8.0 (2 h, 20°C, 1:10 w/v flour/water ratio) was performed. Then, the aqueous dispersion was centrifuged at 10,400 ×g (15 min, 20°C; Beckman Coulter Avanti J25 centrifuge, Beckman Coulter, Fullerton, CA, USA), the pellet was discarded and the supernatant was adjusted to pH 4.5 with 0.1 M HCl for precipitate the storage soy globulins. Soy-whey was separated from the acid aqueous dispersion by centrifugation in the conditions mentioned above.

$SWP_{am-4.5}$ sample was obtained from soy-whey at pH 4.5 by precipitation with ammonium sulfate (90% saturation), centrifugation (12,400 ×g, 15 min, 20°C; Beckman Coulter Avanti J25 centrifuge, Beckman Coulter Inc., Fullerton, CA, USA), dialysis against distilled water (cut-off 12.0 kDa membrane) and subsequent freeze-drying (Sorgentini and Wagner, 1999; Sobral et al., 2010). $SWP_{ac-4.5}$ sample was also obtained from soy-whey at pH 4.5 but by precipitation with acetone previously cooled to -20°C (soy-whey/acetone ratio 1:1 v/v), centrifugation (12,400 ×g, 20 min, 0°C; Beckman Coulter Avanti J25 centrifuge, Beckman Coulter Inc., Fullerton, CA, USA), dialysis against distilled water (cut-off 12.0 kDa membrane) and finally, freeze-drying (Sobral et al., 2010; Sobral and Wagner, 2009). $SWP_{am-8.0}$ and $SWP_{ac-8.0}$ were prepared using the same procedure (precipitation with ammonium sulfate and cold acetone, respectively) from soy-whey previously adjusted to pH 8.0 with NaOH 1.0 M.

Chemical composition

Crude protein content was determined by Kjeldahl method (N×6.25). Carbohydrates content was determined by the phenol-sulfuric acid method (Dubois et al., 1956). In preparing the samples, an exhaustive dialysis step was performed. Hence, the detected carbohydrates are totally, polysaccharides. Dry matter and moisture was determined by heating the samples for 3 h at 103 ± 2°C to constant weight, using a forced draft oven (Memmert; Schwabach, Germany). The determination of total minerals was carried out by dry-ashing at 550 ± 10°C. To determine calcium content, ash was dissolved in 0.14 M nitric acid and 0.5% w/v lanthanum and filtered through a membrane (0.45 μm pore size). Calcium content was determined by atomic absorption spectroscopy using a Shimadzu AA-6650 spectrophotometer (Shimadzu Corporation; Duisburg, Germany) at 422.7 nm (Sobral et al., 2010). All parameters were expressed in grams per 100 g dry weight (% w/w).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoresis was performed on a discontinuous buffered system according to the method of Laemmli (1970) using 12.6% w/v separating gel and 5.0% w/v stacking gel (Bio-Rad, Mini Protean III model, Bio-Rad Laboratories, Inc.; Hercules, CA, USA). Aqueous dispersions of samples were mixed with an equal volume of sample buffer (0.25 M Tris-HCl, 2.0% w/v SDS, 10.0% v/v glycerol and 0.05% w/v bromophenol blue) in the absence and in the presence of 5.0% v/v 2-mercaptoethanol (2-ME). Samples were heated in boiling

water for 5 min, cooled at room temperature and centrifuged (12,100 ×g, 4°C, 20 min; Beckman Coulter GS-15Rcentrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Aliquots of samples containing 50 μg of protein were applied to each gel slot. Gels were dyed with Coomasie Blue R-250 (0.2% w/v in ethanol : water : acetic acid, 40:40:20% v/v/v) and finally, distained (water : ethanol : acetic acid 65 : 25 : 10% v/v/v).

Trypsin inhibitor activity

Trypsin inhibitor activity (TIA) was determined according to the method reported by Sobral and Wagner (2009). The inhibition of porcine trypsin activity on buffered hemoglobin solution (pH 8.0) was evaluated by addition of aqueous dispersion of samples. TIA was expressed as relative TIA (RTIA %) with respect to that of the $SWP_{am-8.0}$ sample (RTIA = 100%).

Differential scanning calorimetry (DSC)

Sample dispersions (30% w/w in distilled water) were hermetically sealed in standard aluminum pans. Thermograms were obtained at 5°C/min heating rate in the range of 30-120°C using a calorimeter (TA Q-200 model, TA Instruments Waters LLC; New Castle, DE, USA). An empty pan was used as reference. From thermograms, peak temperature (Tp, °C) and total denaturation enthalpy (ΔH_d, J/g protein) were obtained.

Protein solubility (S_P)

Aqueous dispersions (0.1 and 1.0% w/v) were prepared by dispersing the samples in 10 mM sodium phosphate buffer pH 7.0. All samples were subsequently centrifuged (9,300 ×g, 20 min, 20°C; Beckman Coulter GS-15Rcentrifuge, Beckman Coulter Inc., Fullerton, CA, USA). Protein concentration was determined both in the total dispersions and in supernatants by the Kjeldahl method (N×6.25). S_P % was calculated as:

$$Sp\ (\%) = (P_S/P_T)\times 100 \qquad (1)$$

P_S and P_T are the crude protein concentration (mg/ml) in the supernatants and total dispersions, respectively. S_P was also determined on thermally treated aqueous dispersions. Samples were previously heated at 100°C for 5 min and rapidly cooled to room temperature in an ice-water bath.

Fourier transform infrared spectroscopy by attenuated total reflection (ATR-FTIR)

FTIR spectra were obtained with a Shimadzu IR Affinity-1 model spectrophotometer (Shimadzu Scientific Instruments, Columbia, Maryland, USA) over the range 800-1800 cm^{-1}. The measurement conditions were the following: Happ-Genzel apodization, number of scans = 30 and resolution = 4 cm^{-1}. Aqueous dispersions (1.0% protein w/v, 10 mM sodium phosphate buffer, pH 7.0) were measured without further treatments or previously heated (100°C, 5 min). 10 μL of dispersions were placed into ATR-8200HA device and dried using warm air (<50°C). Environmental background was measured prior to assay each sample. In order to analyze protein conformation, the deconvolution of FTIR spectra was performed. From the average intensity values of the main peaks, the relative percentage (%) of each secondary dominant structure was estimated. Measurements were performed at least in triplicate.

Intrinsic fluorescence and surface hydrophobicity

Intrinsic fluorescence and surface hydrophobicity (H_0) were evaluated on unheated and heated (100°C, 5 min) aqueous dispersions (10 mM sodium phosphate buffer, pH 7.0) after centrifugation (9,300 ×g, 20 min, 20°C; Beckman Coulter GS-15R centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The protein concentration was 0.1% w/v. Intrinsic fluorescence spectra were registered at 25°C over the range 300-400 nm (λ_{exc}= 291 nm) in an Aminco-Bowman Serie 2 spectrofluorometer (Thermo Fisher Scientific; Waltham, Massachusetts, USA). The fluorescence intensity obtained at the maximum emission wavelength (FI_{max} and $\lambda_{em,max,}$ respectively) was registered.

In order to determine H_0, ANS was used as fluorescent probe according to Kato and Nakai method (Kato and Nakai, 1980). The fluorescence intensity of samples containing 3 mL of 0.04 mM ANS and consecutive aggregates (10 μL) of 0.1% w/v of the protein aqueous dispersion before and after centrifugation (9,300 ×g, 20 min, 20°C; Beckman Coulter GS-15Rcentrifuge, Beckman Coulter Inc., Fullerton, CA, USA) were registered (FI_b). The fluorescence intensity was also determined in samples containing protein without the addition of the fluorescent probe (FI_p). λ_{exc} and λ_{em} were 396 and 478 nm, respectively and temperature was set to 25°C. For total and soluble fractions of samples, H_0 was determined as the initial slope from the plot of net values of fluorescence intensity (FI_b–FI_p) as a function of protein concentration.

Surface behavior at the air/water interface

The surface properties of protein samples at the air/water interface were evaluated on unheated and heated

Table 1. Chemical composition, thermal behavior (Peak temperature, Tp and total denaturation enthalpy, ΔH_d) and relative trypsin inhibitor activity (RTIA) of soy-whey proteins. RTIA (%) = 100 corresponds to 73.4% inhibition of trypsin activity.

Sample	Chemical composition (% w/w as dry basis)				Thermal behavior			RTIA (%)
	Crude protein (N×6.25)	Polysaccharides	Ash	Calcium	Tp (°C) peak I	Tp (°C) peak II	ΔH_d (J/g protein)	
$SWP_{am-8.0}$	98.2±0.3[a]	0.7±0.2[a]	0.40±0.05[a]	0.064±0.005[a]	80.8±0.5[a]	91.7±0.5[a]	10.1±0.5[a]	100
$SWP_{am-4.5}$	89.4±1.2[b]	2.0±0.2[b]	0.91±0.07[a]	0.109±0.005[b]	76.8±0.6[b]	85.6±0.6[b]	7.6±0.5[b]	98.7±2.3[a]
$SWP_{ac-8.0}$	56.7±1.7[c]	25.6±0.8[c]	9.42±0.84[b]	0.520±0.007[c]	79.1±0.2[c]	93.9±0.3[c]	10.7±0.4[a]	92.4±5.4[a,b]
$SWP_{ac-4.5}$	54.2±2.1[c]	27.2±0.7[d]	11.20±0.79[b]	1.090±0.011[d]	76.2± 0.3[b]	87.2±0.4[d]	8.2±0.3[b]	90.5±4.5[b]

Means within the same column following by different letters are significantly different ($p<0.05$), as determined by Fisher's test.

(100°C, 5 min) aqueous dispersions (10 mM sodium phosphate buffer, pH 7.0) before and after centrifugation (9,300 ×g, 20 min, 20°C; Beckman Coulter GS-15Rcentrifuge, Beckman Coulter Inc., Fullerton, CA, USA) at 0.1% w/v protein concentration. The surface tension (γ) was measured at 20°C using an automated drop volume tensiometer (Lauda TVT 2; Lauda-Königshofen, GmbH and Co., Germany). γ was calculated by using the Gauss–Laplace equation:

$$\gamma = [(\Delta\rho \times g \times V)/(2\pi \times r_{cap} \times f)] \quad (2)$$

where $\Delta\rho$ is the density difference of the two adjacent phases, g is the acceleration constant, V is the drop volume, r_{cap} is the outer radius of capillary and f is a correction function necessary to relate the surface tension to the volume of detached drop (Miller et al., 1992).

The surface pressure $(\Pi_{(t)})$ values were recorded as a function of time. $\Pi_{(t)}$ was calculated as:

$$\Pi_{(\tau)} = \gamma_0 - \gamma_{(\tau)} \quad (3)$$

Where γ_0 is the surface tension of 10 mM sodium phosphate buffer (~ 73.0 mN/m) and $\gamma_{(\tau)}$ is the surface tension of aqueous dispersions at different times. It is known that the surface pressure after short times of adsorption is an indicative parameter of the diffusion rate at the air/water interface (Wagner and Gueguen, 1995). Hence, in order to evaluate the diffusion rate in the first step of adsorption, the initial surface pressure (Π_i, mN/m) was calculated at t=3s. In addition, the equilibrium surface pressure (Π_e, mN/m) was obtained at the time where a minimum constant γ value was reached (t > 400 s).

Statistical analysis

Data were analyzed by ANOVA, and significant difference between the Fisher's test was determined (Systat, 5.0; Systat, Point Richmond; CA, USA). The significance level was set at 0.05. Each value reported was the mean of at least three determinations and expressed as such with standard deviation.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of all samples is shown in Table 1. $SWP_{am-4.5}$ exhibited a protein content of ~90% w/w and polysaccharides and ash contents were relatively low. $SWP_{am-8.0}$ showed the highest protein content ($p<0.05$). Although soy-whey is particularly rich in soluble polysaccharides (Espinoza-Martos and Rupérez, 2006; Sobral and Wagner, 2007), a low polysaccharides content in both SWP_{am} samples was observed in comparison with that of SWP_{ac} ones.

This result would be attributed to the specific precipitation of proteins by salting-out. However, the sample $SWP_{am-4.5}$ evidenced a slightly higher polysaccharide content than that of $SWP_{am-8.0}$ ($p<0.05$), probably because at the isoelectric pH there is a greater interaction between proteins and polysaccharides than at alkaline pH. Indeed, salts that are involved in this interaction cannot be totally removed in the final stage of dialysis. Hence, $SWP_{am-4.5}$ evidenced significant higher contents of ash and calcium respect to that prepared from soy-whey at pH 8.0 ($p<0.05$). In addition, both samples obtained by treatment with cold acetone exhibited a protein content lower than 60% w/w. This fact could be attributed to concomitant precipitation of proteins, polysaccharides and minerals induced by the organic solvent. Thus, high values of polysaccharides and ash contents were observed for $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ samples. These results could be due to the fact that the solvating power of water for hydrophilic molecules and ions decreases in the presence of water-miscible organic solvents such as acetone (Scopes,

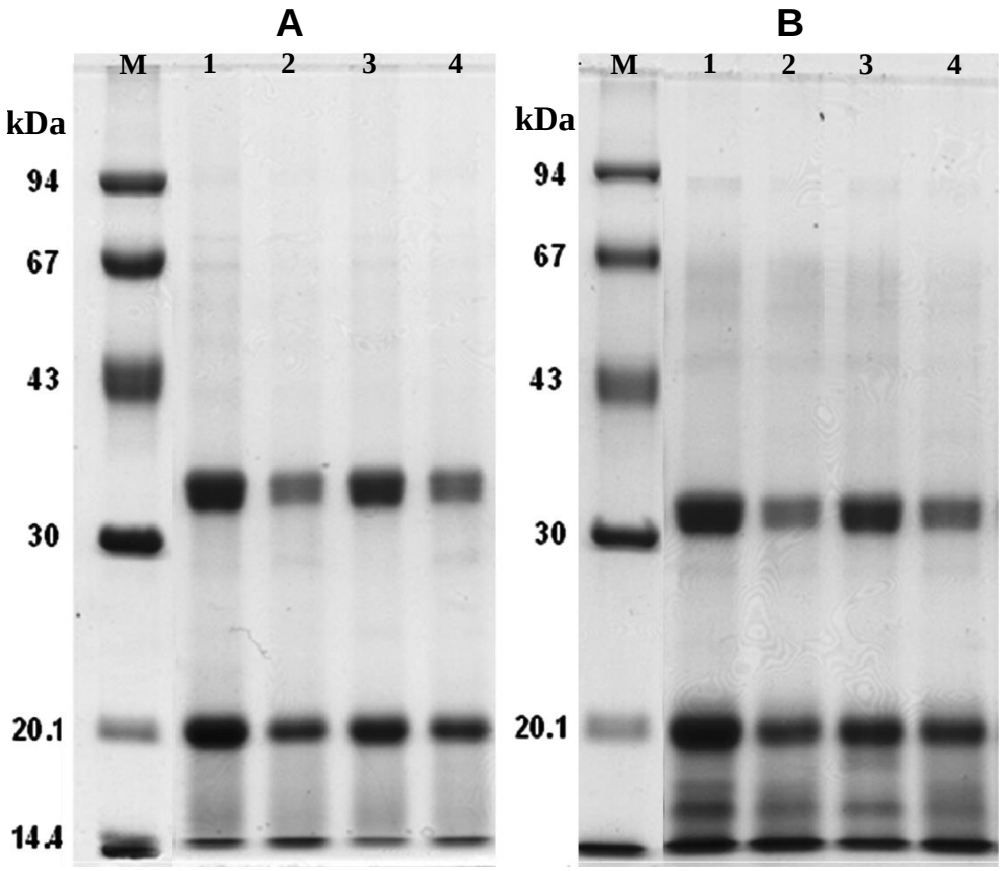

Figure 1. Electrophoretic patterns (SDS-PAGE) of soy-whey proteins: **A,** in non-reducing; **B,** reducing conditions (2-mercapthoethanol, 2-ME); Lanes: **M,** molecular mass markers; **1,** $SWP_{ac-4.5}$; **2,** $SWP_{am-4.5}$; **3,** $SWP_{ac-8.0}$; **4,** $SWP_{am-8.0}$.

1994).

In addition, the electrophoretic patterns of soy-whey samples, in the absence (Figure 1A) and presence (Figure 1B) of 2-ME, were fairly similar (lines 1 to 4). The main bands at ~35 and 20 kDa would be attributed to lectin subunit and KTI, respectively (Ray and Rousseau, 2013). In the presence of 2-ME, some bands in the range 14.4-20 kDa were also observed, which would correspond to some polypeptides from the partial dissociation of insoluble aggregates. Moreover, both in non-reducing and in reducing conditions, no bands were observed at the end of the stacking gel. This would be consistent with the fact that the aggregates of very high molecular mass, initially present in the samples, were partially dissociated in the presence of SDS or 2-ME, generating the low molecular polypeptides of 14-20 kDa.

Thermal behavior and antitryptic activity

DSC thermograms of aqueous dispersions of soy-whey proteins exhibited two endothermic transitions corresponding to KTI and lectin denaturation (endothermic transitions I and II, respectively) (Sobral et al., 2010). Peak temperatures of I (Tp I) and II (Tp II) transitions and total denaturation enthalpies (ΔH_d) are shown in Table 1.

Tp and ΔH_d values for soy-whey proteins were found to be highly dependent on preparation conditions. The thermal stability of KTI and lectin was reduced when the precipitation was made in acidic medium because a lower Tp was obtained for $SWP_{am-4.5}$ and $SWP_{ac-4.5}$ samples ($p<0.05$). At the same time, for samples precipitated at equivalent pH, a higher Tp II value indicates that acetone would promote a thermal stabilization of KTI.

A comparative analysis of ΔH_d values of samples revealed that a similar degree of protein denaturation was observed with both precipitants, regardless the pH during precipitation step. In contrast, the protein insolubilization from soy-whey without previous neutralization (pH 4.5) enhanced the protein denaturation whatever the precipitant used.

On the other hand, all samples exhibited antitryptic activity (Table 1). The highest degree of trypsin inhibition

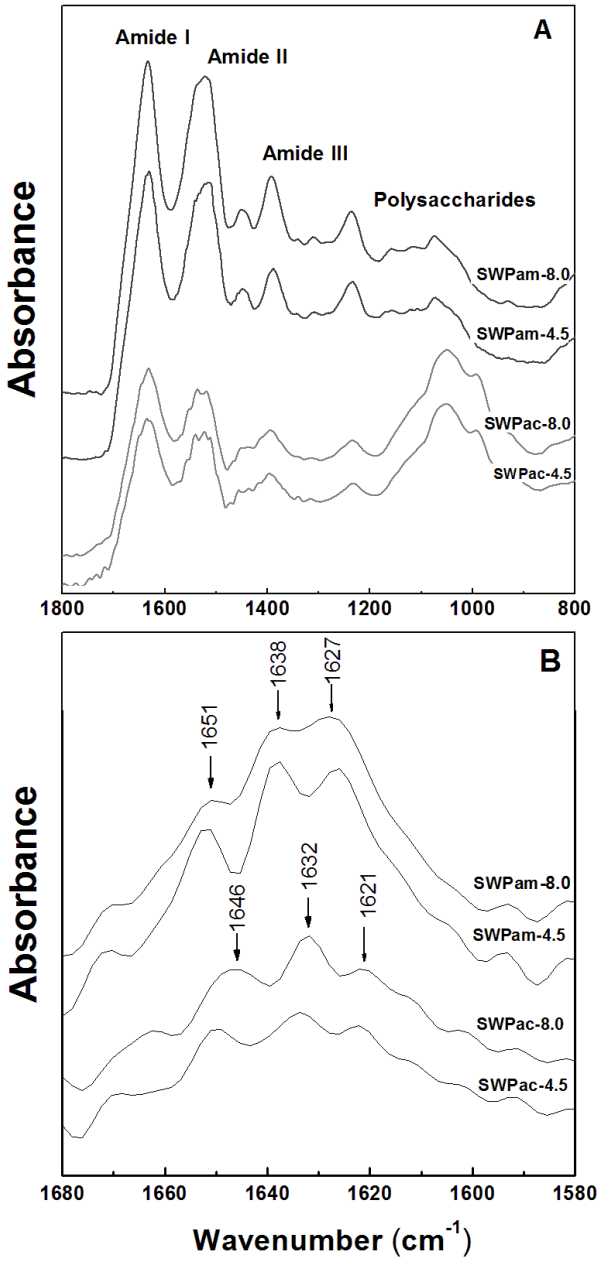

Figure 2. A, ATR-FTIR spectra (800-1800 cm^{-1}) of soy-whey proteins; **B,** Deconvolved FTIR spectra corresponding to Amide I zone (1580-1680 cm^{-1}).

was obtained for $SWP_{am-8.0}$ and hence, this sample was taken as reference (RTIA = 100%). On this basis, all the other, samples showed RTIA values > 90%. Interestingly, no correlation was evidenced between RTIA and ΔH_d values. The integrity of the biologically active site for trypsin inhibitors was kept even though some degree of protein denaturation was evidenced in the samples. Therefore, the protein denaturation associated to the method of preparation of samples not necessarily implies a loss of their antitryptic activities.

FTIR and S_P

FTIR spectra of total, soluble and insoluble fractions for each sample, with and without previous heating (100°C, 5 min) were registered in the range 800-1800 cm^{-1}, where carbohydrate and protein typical bands were found (Khajehpour et al., 2006).

In the 1400-1700 cm^{-1} range, $SWP_{am-8.0}$ and $SWP_{am-4.5}$ samples exhibited the typical amide I (AI), II (AII) and III (AIII) bands (Figure 2A). In addition, all samples also exhibited absorption bands in the range 980-1070 cm^{-1}, which correspond to polysaccharides fingerprint region (PS) (Černá et al., 2003). The band at 978-981 cm^{-1} located within this region almost disappeared for the samples prepared by salting-out. This observation was in agreement with their low polysaccharides contents (Table 1).

To determine the secondary structure of proteins based on FTIR spectra, the deconvolved amide I and amide III regions have been widely used by numerous researchers (Chen et al., 2013; Singh, 1999). The deconvolved spectra of all samples did not show any differences in the AIII zone (data not shown). In contrast, some spectral differences were evidenced in the AI zone (Figure 2B). For $SWP_{am-8.0}$ and $SWP_{am-4.5}$ samples, the IR spectrum displayed a high-intensity AI bands at 1628-1637, 1651 and 1670 cm^{-1}, which were attributed to β-sheet, α-helix/random coil and β-turn structures, respectively (Singh, 1999). The relative intensities of these peaks showed a predominance of β-sheet over the other structures for both samples: 42-44% β-sheet, 34-35% α-helix/random coil and 21-22% β-turn. According to Azarkan et al. (2006), β-sheet is the main secondary structure of KTI. For lectin, β-sheet and in a lesser extent, α-helix are the predominant structures (Sharon and Lis, 1999).

On the other hand, $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ samples exhibited main AI peaks at 1622-1631 cm^{-1} (β-sheet), 1647 cm^{-1} (α-helix/random coil) and 1662 cm^{-1} (β-turn), with a similar preponderance of the first two structures (35-38%) and 24-26% for β-turn. These results would indicate that the process of precipitation of soy-whey proteins would have a clear effect on the secondary structure of the obtained samples, but not the pH of the starting soy-whey. In addition, the precipitation with cold acetone would promote a secondary structure slightly more disordered than that achieved with precipitation by salting out.

With regard to the FTIR spectra of thermally treated samples, $SWP_{am-4.5}$ and $SWP_{am-8.0}$ evidenced similar

Table 2. S_P values of soy-whey proteins. S_P was expressed as (mg of soluble protein/mg of total protein) × 100. Measurements were performed at two sample concentrations: 0.1 and 1.0% w/v in 10 mM sodium phosphate buffer, pH 7.0. S_P was determined on samples without thermal treatment and after heating at 100°C for 5 min.

Samples	Without heating	With previous heating
	0.1%	
$SWP_{am-8.0}$	94.5±1.2 [a, 1]	91.4±1.7 [a, 2]
$SWP_{am-4.5}$	88.8±1.0 [b, 1]	88.2±1.2 [a, 1]
$SWP_{ac-8.0}$	88.1±0.7 [b, 1]	86.4±3.1 [a, 1]
$SWP_{ac-4.5}$	86.5±0.9 [c, 1]	77.5±3.6 [b, 2]
	1.0%	
$SWP_{am-8.0}$	93.1±1.9 [a, 1]	87.6±1.7 [a, 2]
$SWP_{am-4.5}$	85.5±1.2 [b, 1]	79.4±1.2 [b, 2]
$SWP_{ac-8.0}$	87.4±2.0 [b, 1]	81.5±2.2 [b, 2]
$SWP_{ac-4.5}$	85.4±1.3 [b, 1]	72.2±1.6 [c, 2]

Means within the same column followed by different lowercase letters are significantly different, as determined by the Fisher's test ($p<0.05$). Means within the same file followed by different numbers are significantly different, as determined by the Fisher's test ($p<0.05$).

secondary structures respect to those of unheated samples, with a predominance of β-sheet (42-47%). $SWP_{ac-4.5}$ and $SWP_{ac-8.0}$ exhibited mainly a α-helix/random coil structure (43%) and, in a lesser extent, β-sheet (30-40%) and β-turn (21-26%). This result would indicate that the thermal denaturation of samples prepared by precipitation with acetone increases the proportion of disordered structures.

As mentioned above, the polysaccharide content of soy-whey proteins obtained by precipitation with acetone was fairly high (Table 1). This result was in clear agreement with the relative low ratio between the absorbance of main AI band and that of polysaccharides (1050-1080 cm^{-1}) (AI/PS) for $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ samples. For these samples AI/PS ratio was ~1 while for those prepared by salting out, it was higher than 1.6.

The S_P values for all samples are shown in Table 2. Broadly, soy-whey samples exhibited different S_P, in accordance with the state of protein aggregation, which directly depended on preparation methods. Besides, the higher the sample concentration, the lower S_P. These results would indicate that insoluble protein aggregates can be effectively dissociated upon dilution. At both sample concentrations, S_P decreased as follows: $SWP_{am-8.0}$>$SWP_{am-4.5}$~$SWP_{ac-8.0}$>$SWP_{ac-4.5}$ ($p<0.05$). At both sample concentrations (0.1 and 1.0% w/v), the protein samples obtained by precipitation with acetone showed a higher state of protein aggregation respect to those obtained by salting-out. This result was especially evident for samples obtained from soy-whey at pH 8.0 ($p<0.05$).

However, at 1.0% w/v, S_P did not show significant differences between $SWP_{ac-4.5}$ and $SWP_{am-4.5}$ samples ($p>0.05$). At the same time, for samples prepared with the same precipitation method, a lower S_P was effectively observed at pH 4.5. At both sample concentrations, the differences in S_P were more noticeable for $SWP_{am-8.0}$ and $SWP_{am-4.5}$ samples ($p<0.05$) (Table 2).

Moreover, all heated samples evidenced an additional degree of protein aggregation (lower S_P values), especially at high sample concentration, where significant differences of S_P values were effectively observed between unheated and heated samples ($p<0.05$) (Table 2). This fact is not surprising. Heat-induced protein aggregation requires interactions between polypeptide molecules and hence, it is always favored at a high sample concentration (Zayas, 1997).

As mentioned above, the concomitant precipitation of polysaccharides and proteins was proposed for samples obtained by precipitation with cold acetone. However, the determination of S_P provides no information about the relative carbohydrate and protein composition of the soluble and insoluble fractions. To test this, FTIR spectra of both fractions were comparatively examined, and the AI/PS ratio was obtained for unheated and heated samples (Figures 3A and B).

Except for $SWP_{am-8.0}$ sample, which exhibit a very low carbohydrate content (<1% w/w, Table 1), the insoluble fraction of the unheated samples contains a greater proportion of proteins than that of soluble fractions (Figure 3A). This is observed for $SWP_{am-4.5}$ and especially for $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ samples, although their carbohydrate contents were fairly high (Table 1). This result indicates that the aggregates were composed mainly by proteins. Moreover, the differences observed in the AI band spectral position (AIp), between soluble and insoluble fractions, could be directly attributed to structural modifications. For $SWP_{am-8.0}$ and $SWP_{am-4.5}$ samples, AIp values were always higher in the insoluble fraction ($p<0.05$). According to Carbonaro and Nucara (2010), frequencies with an average of 1645 cm^{-1} would indicate an increase in random coil structure for insoluble proteins. On the other hand, for samples precipitated with acetone, there were no differences between the AIp values of the soluble and insoluble fractions ($p>0.05$).

In addition, heating produced a significant increase in AI/PS ratio only for insoluble fractions of $SWP_{ac-4.5}$ and $SWP_{ac-8.0}$ samples (Figure 3B). As mentioned above, this result is consistent with an enrichment of aggregated proteins even though the aqueous dispersions contain relatively high amount of polysaccharides. $SWP_{am-4.5}$, $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ exhibited a significant increase of AIp values in the soluble fraction while an opposite trend was observed for their insoluble fraction ($p<0.05$). This increase in the differences of AIp values between both fractions after heating (~20 cm^{-1}) would be attributed to conformational changes associated to an advanced

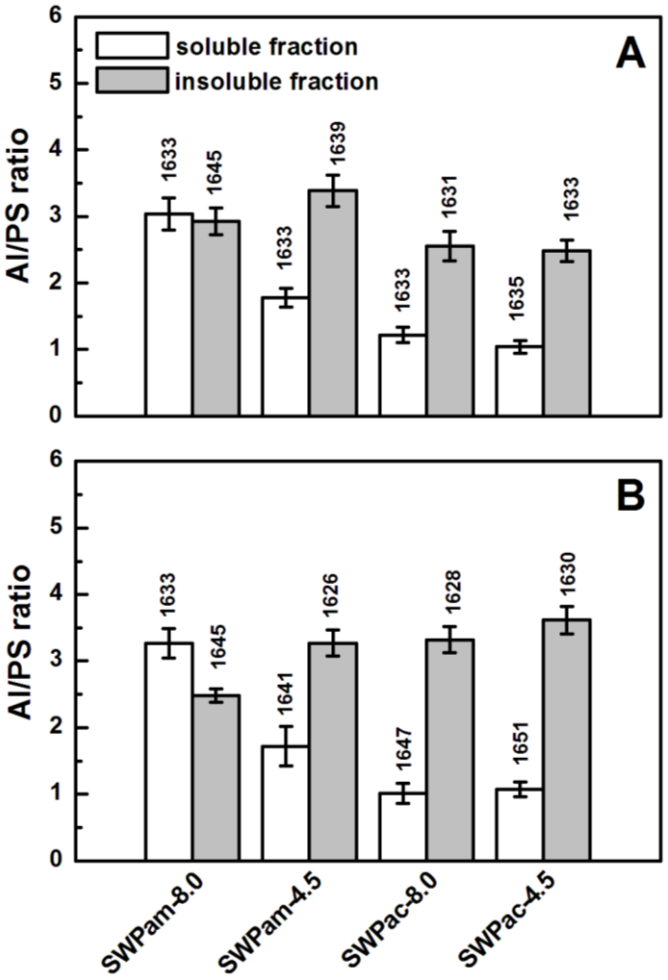

Figure 3. Numerical ratio (AI/PS) between the maximum absorbance values of Amide I and polysaccharides (1050-1080 cm^{-1}) peaks corresponding to FTIR spectra of soluble and insoluble fractions of soy-whey proteins. **A,** Unheated; **B,** heated (100°C, 5 min) samples. Numbers above the bars indicate the spectral position (wavenumber, cm^{-1}) of maximum Amide I peak.

degree of protein aggregation in the insoluble fraction plus an interaction between denatured proteins and polysaccharides in the soluble fraction (Carbonaro and Nucara, 2010). The amide I band with an average frequency in the range 1626-1630 cm^{-1} would be consistent with both a structure α-helix/β-turn (Murayama and Tomida, 2004) and an increase in the β type structures due to protein aggregation (Fink et al., 2000).

Fluorescence studies

Here, the fluorescence studies on all samples are assessed though the evaluation of intrinsic fluorescence and aromatic surface hydrophobicity (H_0). Firstly, the intrinsic fluorescence spectra of samples were different regardless the thermal treatment (data not shown). The $\lambda_{em,max}$ suffers a red shift when chromophores become more exposed to the solvent, and the quantum yield of fluorescence (ϕ_F) decreases when the chromophores interact with quenchers either in a solvent or in the protein itself (Shen and Tang, 2012). FI_{max} values, directly associated to ϕ_F and $\lambda_{em,max}$, are summarized in Table 3.

Unheated $SWP_{am-8.0}$ and $SWP_{ac-8.0}$ exhibited lower values of $\lambda_{em,max}$ and concomitantly, higher values of FI_{max} with respect to those precipitated at pH 4.5. These latter samples would present a lower degree of denaturation (Table 1) and hence, these $\lambda_{em,max}$ values are in agreement with partially exposed Trp residues on the protein surface (Eftink, 2000). Moreover, for $SWP_{ac-4.5}$ and $SWP_{ac-8.0}$ samples higher FI_{max} values and a predominant slight blue shift of $\lambda_{em,max}$ ones were effectively observed. This result would be also related to protein denaturation and further formation of protein aggregates, especially after thermal treatment. After heating, a significant increase of FI_{max} was evidenced in all samples, but more markedly for $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ samples ($p<0.05$). Although thermal treatment produced a total protein denaturation in all samples, the structural changes generated by this treatment would be affected by the initial structural and compositional characteristics of the samples. The high value of FI_{max} reached in heated SWP_{ac} samples, especially for $SWP_{ac-4.5}$, would be related to the more disordered structure detected by FTIR. The high content of polysaccharides and calcium (Table 1) would stabilize more disordered structures during heating, allowing a greater exposure of Trp residues (Eftink, 2000).

On the other hand, it is worth noting that H_0 of total and soluble fractions were, in some cases, substantially different (Table 3). Undoubtedly, the presence of the insoluble fraction has a noticeable impact on H_0. In addition, during H_0 measurements, the diluted dispersions were completely limpid. This would be related to the fact that insoluble aggregates are partially or totally converted into soluble aggregates upon the high dilution during the H_0 measurement (Zhu et al., 2017). Therefore, it would be important to take into consideration the contribution of insoluble fraction to global H_0 ($H_{0,I}$), which could be estimated as follows:

$$H_{0,T} = S_P \times H_{0,S} + (1 - S_P) \times H_{0,I} \quad (4)$$

$$H_{0,I} = [(H_{0,T} - S_P \times H_{0,S}) / (1 - S_P)] \quad (5)$$

$H_{0,T}$ and $H_{0,S}$ are the H_0 values for total and soluble fraction, respectively, and S_P is protein solubility expressed in g/ml (Table 2). Soy-whey proteins exhibited different values of $H_{0,I}$ as a function of pH during the preparation. For the samples obtained at pH 8.0, the

Table 3. Maximum fluorescence intensity (FI_{max}), fluorescence emission maximum ($\lambda_{em,max}$ in parenthesis) and aromatic surface hydrophobicity (H_0) for total ($H_{0,T}$) and soluble ($H_{0,S}$) fraction of unheated and heated (100°C, 5 min) soy-whey protein samples. Theoretical $H_{0,I}$ values were estimated from $H_{0,T}$ and $H_{0,S}$ ones using the Equation 5.

Samples	Heating	FI_{max} ($\lambda_{em,max}$) ♦	H_0 experimental (mg/g)$^{-1}$ •		H_0 theoretical
			Total ($H_{0,T}$)	Soluble ($H_{0,S}$)	Insoluble ($H_{0,I}$)
$SWP_{am-8.0}$	–	2.43 ± 0.08 ^{a} (337)	53.4 ± 0.1^{a,1}	51.6 ± 0.1^{a,1}	77.7
$SWP_{am-4.5}$		1.66 ± 0.06 ^{b} (342)	69.1 ± 0.1^{b,1}	33.4 ± 0.1^{b,2}	279.6
$SWP_{ac-8.0}$		3.90 ± 0.10 ^{c} (338)	16.8 ± 0.1^{c,1}	13.5 ± 0.1^{c,1}	39.9
$SWP_{ac-4.5}$		2.61 ± 0.05 ^{a} (340)	53.8 ± 0.4^{a,1}	31.6 ± 0.1^{b,2}	183.6
$SWP_{am-8.0}$	+	2.90 ± 0.05 ^{d} (340)	235.1 ± 0.2^{d,1}	250.3 ± 0.6^{e,2}	127.4
$SWP_{am-4.5}$		2.55 ± 0.07 ^{a} (343)	260.7 ± 0.2^{e,1}	155.4 ± 0.2^{f,2}	666.5
$SWP_{ac-8.0}$		6.02 ± 0.12 ^{e} (340)	218.5 ± 0.3^{f,1}	261.0 ± 0.8^{g,2}	31.3
$SWP_{ac-4.5}$		4.50 ± 0.11 ^{f} (342)	304.6 ± 1.2^{g,1}	218.3 ± 0.6^{h,2}	528.7

♦, All values are the mean of three independent determinations (n=3); •, mean values within the same column followed by different letters are significantly different, as determined by Fisher's test ($p < 0.05$).
Mean values within the same line followed by different numbers are significantly different, as determined by Fisher's test ($p < 0.05$).

insoluble fraction contributes with a surface hydrophobicity of the same order as that of the proteins of the soluble fraction. In contrast, in proteins precipitated at pH 4.5, $H_{0,I}$ was almost 5 times higher than in those precipitated at pH 8.0. It must be taken into account that at pH 4.5, near the isoelectric point of KTI (~4.5) and lectin (~5.8), the formation of close-structured aggregates with more hydrophobic proteins was induced (Wang, 1971, Chang et al., 2014). As result of this, for $SWP_{ac-4.5}$ and $SWP_{am-4.5}$ samples, $H_{0,I}$ were 6 to 8 times higher than those of the corresponding soluble fraction ($H_{0,S}$). This result is also in agreement with its higher denaturation degree (Table 1) and a higher contribution of hydrophobic proteins to the formation of insoluble aggregates. As mentioned above, for samples precipitated at pH 4.5, FI_{max} were lower than those obtained at pH 8.0 (Table 3). In this case, an opposite behavior was observed for H_0 measurements. Thus, $SWP_{ac-8.0}$ with the highest FI_{max}, exhibited at the same time, the lowest values of H_0 for both total and soluble fractions.

Moreover, a comparative analysis between soluble and total fractions of heated $SWP_{am-4.5}$ and $SWP_{ac-4.5}$ revealed that $H_{0,T}$ values were fairly higher for the latter samples. In addition, $H_{0,I}$ values of these samples is very high, almost 2.5 times higher than those of the insoluble fractions of the unheated samples. Upon extensive dilution, these aggregates would release occluded hydrophobic sites of proteins. In contrast, for heated $SWP_{am-8.0}$ and $SWP_{ac-8.0}$ samples, H_0 values were always higher in the soluble fraction. These results would suggest that their proteins were aggregated through non-hydrophobic interactions.

According to the exposed results, it is evident that the structural characteristics of both soluble and insoluble proteins of initial samples affect the structural properties of them after thermal treatment. Although the heating was performed in buffer solution at pH 7.0, substantial differences in structural properties were observed between the samples obtained from soy-whey at pH 4.5 and 8.0. In this context, the aggregates formed during the precipitation steps in acid medium, which were composed by proteins of high surface hydrophobicity, would behave as nucleus for the formation of new aggregates during the heating. This hypothesis would be supported by the noticeable higher values of $H_{0,I}$ values respect to $H_{0,S}$ ones for $SWP_{ac-4.5}$ and $SWP_{am-4.5}$ samples. Interestingly, the opposite tendency observed for $SWP_{ac-8.0}$ and $SWP_{am-8.0}$ samples would reinforce the mentioned argument (Table 3).

Surface behavior at the air/water interface

The surface behavior of proteins can be assessed both by the initial (Π_i) as the equilibrium surface pressure (Π_e) parameters. Π_i gives an idea of the rate of adsorption of the protein in the air-water interface and therefore informs about the migration/diffusion process. Moreover, Π_e indicates the effectiveness of the protein to reduce the surface tension once the process of anchorage and rearrangement at the interfacial level was completed (De Feijter and Benjamins, 1987). Broadly, a different surface behavior between unheated and heated samples and between total and soluble fractions was effectively observed in all the samples (Figure 4).

Figure 4A shows that all samples, except for $SWP_{ac-8.0}$, evidenced a noticeable increase of surface pressure after 3 s (Π_i) ($p<0.05$). As was observed by SDS-PAGE, soy-whey protein samples are composed by proteins with relatively low molecular mass (Figure 1). These samples

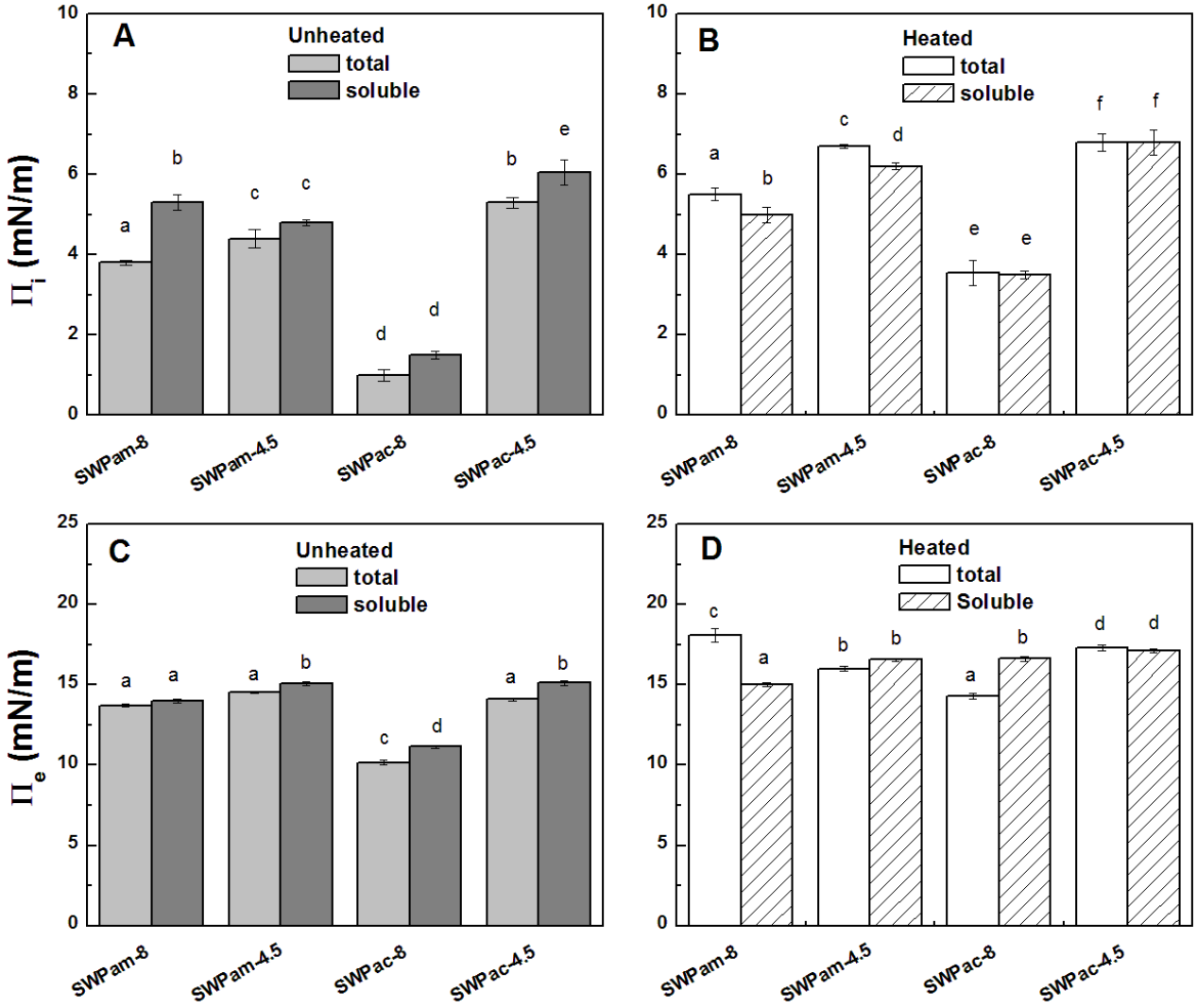

Figure 4. Initial (Π_i) (A, B) and equilibrium surface pressure (Π_e) (C, D) for total and soluble fraction of soy-whey proteins. Unheated (A, C) and heated (100°C, 5 min) samples (B, D). For both parameters different characters on bars represent significant difference at $p<0.05$.

were capable of a quick diffusion toward the interface, and at the same time a quick exposition of their hydrophobic residues to the air phase. In this context, the lowest Π_i values for $SWP_{ac\text{-}8.0}$ sample would be attributed to its noticeably low surface hydrophobicity (H_0 < 20, Table 3). In addition, Figure 4A shows that in most cases, the soluble fractions exhibited higher Π_i values respect to those of total fractions. These results are in agreement with those reported by Maticorena et al. (2018) on the surface behavior of whey protein isolated with different protein aggregation degree. They report a slight effect of the degree of aggregation over diffusion, which indicate that native and non-aggregated proteins dominated the decrease in surface tension at short time.

According to Figure 4B, thermal treatment of sample dispersions (100°C, 5 min) of total fractions significantly promote an increase of Π_i, which would be associated to an increase of H_0 (Table 3, $p<0.05$). Unlike the behavior observed for unheated $SWP_{am\text{-}8.0}$ and $SWP_{am\text{-}4.5}$ samples, after thermal treatment, the Π_i values of total fraction were higher than those of soluble fraction ($p<0.05$). These results would be consistent with the high surface activity of protein aggregates or alternatively, the dissociation of these protein aggregates in close proximity to the air/water interface (Mahmoudi et al., 2011). The mentioned aggregates would release proteins that are rapidly absorbed.

In the last step of adsorption kinetics, the comparative

analysis between equilibrium surface pressure (Π_e) for unheated samples gives a similar tendency respect to that of Π_I values (Figures 4A and C). Although the polysaccharides and mineral contents were markedly different for $SWP_{am-4.5}$ and $SWP_{ac-4.5}$ samples, they exhibited similar values of Π_e ($p>0.05$). Although, according to Lad et al. (2006), the β-sheet structure favors the absorption and anchoring of the protein at the air/water interface, this result indicates that the surface behavior of these samples would be more affected by the degree of protein denaturation and surface hydrophobicity (Tables 1 and 3).

Figure 4D shows that the thermal treatment significantly improved the surface activity of all samples, presumably by the increase of H_0 (Table 3). This result was in clear agreement with those reported in previous works (Kato and Nakai, 1980; Wagner and Gueguen, 1995). Moreover, the analysis of surface behavior of $SWP_{am-8.0}$ sample revealed that the heated total fraction exhibited a higher Π_e value respect to that of soluble fraction ($p<0.05$). At the same time, for heated samples all soluble fractions showed similar Π_e values, being the differences lower than 2 mN/m ($p<0.05$). Hence, the differences in the surface behavior of total fractions would be attributed to presence of insoluble aggregates, which had a positive effect for $SWP_{am-8.0}$ but it was negative for $SWP_{ac-8.0}$ sample. The nature of the aggregates formed by precipitation at pH 8.0 by salting out would have a less compact and more hydrated structure, due to their relatively low surface hydrophobicity (Table 3). Thus, these aggregates therefore would present a greater contribution to the interfacial activity respect to those of samples precipitated with acetone. In this context, more experimental evidence would be required to support the mentioned hypothesis. In contrast, for samples obtained by precipitation at pH 4.5, regardless the precipitant, the surface behavior was not almost affected by the presence of protein aggregates, as revealed by the comparative analysis of Π_e values of soluble and total fractions ($p<0.05$). In this case, the insoluble aggregates did not affect the surface behavior of soluble proteins. As was evidenced in Table 3, the insoluble aggregates of heated $SWP_{ac-8.0}$ and $SWP_{ac-4.5}$ have a noticeable high surface hydrophobicity; hence, they should be efficiently adsorbed at the air/water interface. Nevertheless, the high surface hydrophobicity would promote a strong interaction between protein molecules reducing their dissociation and interfacial adsorption.

Conclusion

This paper revealed that the structural and surface properties of soy-whey protein samples were markedly dependent on the precipitant and pH of soy-whey during precipitation step. Although the polypeptide composition was similar for all samples, the precipitant mainly affected the proximate composition and, in a lesser extent, the degree of protein aggregation. At the same time, the pH of precipitation had a marked influence on the degree of protein denaturation, the exposure of hydrophobic zones of the protein molecules and the nature of aggregates. The heating of soy-whey proteins, a necessary step to inactivate the Kunitz trypsin inhibitor and lectin, enhanced the surface activity of all samples and the observed differences were attributed to the presence of insoluble aggregates. The results obtained in this paper should be used as basis for the assessment of further studies concerning with the exploitation of soy wastewaters, especially for the isolation of proteins using up-scaling or more industrially-applicable methods.

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