JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Physicochemical Modifications of High-Pressure-Treated Soybean Protein Isolates

CECILIA PUPPO,[†] NICOLAS CHAPLEAU,[‡] FRANCISCO SPERONI,[†] MARIE DE LAMBALLERIE-ANTON,[‡] F. MICHEL,[§] CRISTINA AÑÓN,*[,†] AND MARC ANTON[||]

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) (UNLP - CONICET), 47 y 116, 1900 La Plata, Argentina, Ecole Nationale d'Ingénieurs des Techniques des Industries Agricoles et Alimentaires (ENITIAA), GEPEA, BP 82225, 44322 Nantes Cedex 3, France, Institut National de la Recherche Agronomique, Laboratoire de Recherche et de Technologie Laitière, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France, and Institut National de la Recherche Agronomique, Laboratoire d'Etude des Interactions des Molécules Alimentaires, Groupe Physico-chimie des Emulsions, BP 71627, 44316 Nantes Cedex 3, France

Changes induced by high pressure (HP) treatment (200−600 MPa) on soybean protein isolates (SPI) at pH 3 (SPI3) and pH 8 (SPI8) were analyzed. Changes in protein solubility, surface hydrophobicity (Ho), and free sulfhydryl content (SH_F) were determined. Protein aggregation and denaturation and changes in secondary structure were also studied. An increase in protein Ho and aggregation, a reduction of free SH, and a partial unfolding of 7S and 11S fractions were observed in HP-treated SPI8. Changes in secondary structure were also detected, which led to a more disordered structure. HP-treated SPI3 was partially denatured and presented insoluble aggregates. A major molecular unfolding, a decrease of thermal stability, and an increase of protein solubility and Ho were also detected. At 400 and 600 MPa, a decrease of the SH_F and a total denaturation were observed.

KEYWORDS: Soybean proteins; high-pressure treatment; protein structure; protein denaturation; protein aggregation

INTRODUCTION

Increasing consumer demand for high quality, minimally processed, additive-free, and microbiologically safe food (*1*) has turned worldwide attention to the potential of high pressure (HP) technology in food processing (*2*, *3*). In addition to preserving foods, HP technology can modify functional properties of food components (*4*). While small molecules such as amino acids, vitamins, and flavor compounds remain unaffected by HP, in contrast to thermal processing, noncovalent bonds of proteins and other large molecules are altered (*5*). It is well-known that pressure has a disruptive effect on the tertiary and quaternary structure of most globular proteins (*6*). At high pressures (above 200 MPa), many proteins tend to unfold, and reassociation of dissociated subunits from oligomers can occur. Under certain conditions, dissociation and/or denaturation induced by HP can be reversible (*7*, *8*). Denaturation of proteins by high pressure differs from heat-induced denaturation, the last being often irreversible because of the breakage of covalent bonds and/or aggregation of unfolded protein (*8*). Although the mechanism by which the protein is denatured by pressure is not yet fully understood, it has been suggested that HP causes changes in protein structure via cleavage of noncovalent bonds (*8*, *9*). Pressure would also induce aggregation due to the formation of intermolecular disulfide bridges via SH/S−S interchange linkages (*9*−*11*).

The use of soybean protein isolates (SPI) as functional ingredients in food formulation is interesting both because of their high functionality and nutritional value. However, the extraction method can affect the functional properties of soy proteins (*12*). Consequently, adequate modifications on SPI may enhance and/or normalize their functional properties, particularly the emulsifying properties. The effect of different common treatments on structural and functional properties of soybean proteins was widely studied (*13*−*21*). The major components of an SPI, 11S (glycinin) and 7S (β-conglycinin) globulins, are affected in a distinct manner by different treatments. It was found that heat treatment (HT) induces dissociation, denaturation, and aggregation of 7S and 11S subunits (*22*, *23*). In contrast, acid treatments lead to denaturation and selective dissociation and unfolding of 11S, with lesser effect on 7S and minimal protein aggregation. (*18*, *20*).

* To whom correspondence should be addressed. Tel./Fax: 54-221-4254853. E-mail: mca@biol.unlp.edu.ar.
[†] CIDCA.
[‡] ENITIAA.
[§] Institut National de la Recherche Agronomique, Laboratoire de Recherche et de Technologie Laitière.
[||] Institut National de la Recherche Agronomique, Laboratoire d'Etude des Interactions des Molécules Alimentaires.

10.1021/jf034813t CCC: $27.50
Published on Web 03/02/2004

Only limited data have been reported about the effect of HP on structural and functional properties of soybean proteins. HP treatment at neutral pH improved the emulsifying activity but not the emulsifying stability of soy proteins (*24*). On the other hand, self-supporting gels of SPI and 7S and 11S fractions were obtained at HP in the range 300−700 MPa with improved water holding capacity (*25*). Little is known about the effect of HP on the molecular structure of soybean proteins. Therefore, the objective of this work was to study the changes induced by HP treatment on the structure and emulsifying properties of soybean proteins at neutral and acidic pH. To this end, we have subjected 1% (w/v) SPI to HP at pH 3 or 8, and measured solubility, free sulfhydryls, surface hydrophobicity, thermal stability, secondary structure, and aggregation of the samples.

MATERIALS AND METHODS

Preparation of Soy Protein Samples. SPI was prepared from defatted flour manufactured by Bunge−Ceval S. A. (Brazil). An alkaline extraction (pH 8.0), followed by precipitation at the isoelectric point (pI = 4.5) was carried out according to Puppo et al. (*17*). The isoelectric precipitate was dispersed in distilled water and adjusted to pH 8.0 with 2 N NaOH. The dispersion thus obtained was lyophilized. Protein content of SPI, determined by the microKjeldahl method using a colorimetric method for protein detection (*26*), was 86.6 ± 1.6% (N × 6.25).

For HP processing, dispersions of SPI protein concentration 10 g/L at pH 8 (50 mM Tris-HCl) (SPI8) and pH 3 (50 mM glycine) (SPI3) were prepared.

Preparation of 7S and 11S Fractions. 7S and 11S globulins were obtained according to the method of Nagano et al. (*27*). The defatted flour was dispersed in distilled water (1:15, w/w), adjusted to pH 8.0 with 2 N NaOH, stirred at room temperature for 2 h and centrifuged at 13 300*g* for 20 min at 15 °C. Dry sodium bisulfite ($NaHSO_3$) was added to supernatant (0.98 g $NaHSO_3$/L), the pH was adjusted to 6.4 with 2 N HCl, and the mixture was kept overnight at 4 °C. The dispersion was centrifuged at 6500*g* for 20 min at 4 °C. The precipitate (11S fraction) was suspended in distilled water, adjusted to pH 7.8 with 2 N NaOH, dialyzed and lyophilized. Salt concentration of supernatant was adjusted with solid NaCl to 0.25 M and pH was adjusted to 5.0 with 2 N HCl. After 1 h, the insoluble fraction was removed by centrifugation (9000*g* × 30 min at 4 °C). The supernatant was diluted 2-fold with cold water and the pH was adjusted to 4.8 with 2 N HCl. Centrifugation at 6500*g* × 20 min at 4 °C was carried out. The washed precipitate (7S fraction) was suspended in distilled water, adjusted to pH 7.5 with 2 N NaOH, and dialyzed before freeze-drying.

High-Pressure Processing. HP processing was carried out in a 1.5 L reactor unit (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure regulation. Prior to pressure processing, SPI, glycinin (11S fraction), and β-conglycinin (7S fraction) dispersions (50 mL) were vacuum-conditioned in a polyethylene bag (La Bovida, France). Temperature during treatment was controlled to avoid freezing or overheating of proteins. Conditions of HP processing were chosen in accordance to Chapleau and de Lamballerie-Anton (*28*). Protein dispersions (1% w/v) were subjected to HP treatment at 200, 400, and 600 ± 7 MPa for 10 min. The target pressure was reached at 6.5 MPa/s and released at 20 MPa/s. The temperature of the transmitting medium in the vessel was kept at 20 ± 2 °C during pressure processing.

Protein Solubility. Treated and nontreated (control) SPI dispersions were centrifuged at 10 000*g* for 20 min at 20 °C. The protein content of the supernatant was determined by the Biuret procedure, using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard. Percent protein solubility was calculated as

$$\text{solubility (\%)} = \text{protein in the supernatant (mg/mL)} \times 100/\text{initial protein (mg/mL)}$$

Free Sulfhydryls. Free sulfhydryls (SH_F) in SPI8 and SPI3 dispersions were determined according to the method of Petruccelli and Añón (*15*). Ellman's reagent was prepared by dissolving 40 mg of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) in 10 mL of methanol. A 1-mL aliquot of each sample was incubated for 45 min at room temperature with 40 μL of the reagent. Absorbance was measured at 412 nm in a UV−vis Perkin-Elmer Lambda 12, 88647 (Uberlingen, Deutschland) spectrophotometer. Results were expressed as the mean of duplicate analysis.

Surface Hydrophobicity. Surface hydrophobicity (H_0) of SPI8 and SPI3 samples were measured according to Kato and Nakai (*29*) using the fluorescent probe 1-anilino-8-naphthalene-sulfonate (ANS). Protein dispersions were diluted (0.04 to 0.2 mg/mL) in the extraction solvent. Then, 10 μL of ANS (8.0 mM in sample buffer) were added to 2 mL of sample. Fluorescence intensity (FI) was measured with a Hitachi F-4500 fluorescence spectrometer (Tokio, Japan) at 380 nm (excitation) and 490 nm (emission) wavelengths. The initial slope of fluorescence intensity versus protein concentration plot was used as an index of H_0. Measurements were performed by triplicates.

Differential Scanning Calorimetry. Differential scanning calorimetry was performed on a Micro DSC III (SETARAM, Caluire, France). Nontreated and HP-treated SPI, 7S, and 11S soybean subunits dispersions at pH3 and pH8 (5% w/w protein-40 mg of dry matter) were heated in the calorimeter from 30 to 110 °C at 1 °C/min. A second scan was carried out on samples to check denaturation reversibility. Samples were hermetically sealed in iron pans. Sample buffer was used as reference. After cooling, sample and reference were heated again under the same conditions to check reversibility. Enthalphies of thermal denaturation were estimated as the area under the DSC curve. The enthalpy value of each peak was calculated by Micro DCS III software and expressed as J/g of protein (dry matter basis). The endothermic peak temperature was calculated using Peak Fit software V4.0 (Jandel Scientific Software, Chicago, IL). Results were the mean of three measurements. Statistical analysis was performed using a one-way analysis of variance according to the general linear model procedure with least-squares mean effects to determine significant differences between treatments. Multiple range test was applied to determine which means were significantly different according to Fisher's least significant differences (LSD). Significant differences of inactivation were determined with 5% level of significance ($P < 0.05$) by Student's test. Statistical analysis was carried out using Statgraphics plus version 2.1 software (Statistical Graphics Corp., Princeton, NJ). The degree of denaturation was calculated as the percentage of protein denaturation compared to native protein at pH 8.

Circular Dichroism (CD). Secondary structure changes of buffer-soluble SPI8 and SPI3 samples were determined by measuring the absorbance of polarized light in the 180−250 far UV range. A quartz cell of 0.01 cm length was used. CD measurements were carried out in a spectropolarimeter Jobin−Yvon CD6 (Jovin−Yvon SA, Longjumeaux, France). Molar ellipticity, θ (deg · cm^2/dmol) was calculated from the average molecular mass of the SPI, the protein concentration of the sample, and the optic length. Percentages of secondary structures were calculated from molar ellipticity using Dicroprot 2000 software (G. Deleage, France) with the Varselec program.

Size Exclusion Chromatography. Centrifuged SPI dispersions (10 000*g* 20 min at 20 °C) were filtered with cellulose acetate filter 0.45 μm (Sartorius AG, Goettingen, Germany). A Sephacryl S-500 (Amersham Biosciences, UK) column (1000- × 16-mm^2) was utilized. Calibration was carried out with BSA (66 kD), alcohol dehydrogenase (150 kD) and thyroglobulin (670 kD). The column buffers were the same as sample buffer. Samples were eluted from the column at a flow rate of 1 mL/min. Chromatography was carried out with an Amersham Pharmacia system, and fractions were detected at 280 nm in a 3-mm quartz-cell. To determine the surface area of each protein fraction, deconvolution of peaks was performed by a Peak Fit software V4.0 (Jandel Scientific Software) according to the Gaussian method.

Quasi-Elastic Light Scattering (QELS). The average diameters of protein particles of SPI8 and SPI3 dispersions were measured by photon correlation spectroscopy (PSC) using a Log−Lin correlator (Malvern Instruments Ltd., Malvern, Worcs. UK). Measurements of the dynamics of scattered light were made at 90°, and average diffusion coefficients were determined by the method of cumulants. Particle diameters were calculated from diffusion coefficients using the Stoke-Einstein relation for spheres.

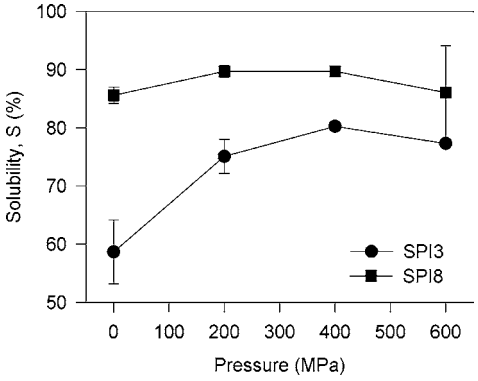

Figure 1. Solubility (S%) of nontreated (0.1 MPa) and HP-treated (200–600 MPa) SPI dispersions (1% w/w). SPI dispersions: acidic-pH 3 (SPI3) and alkaline-pH 8 (SPI8).

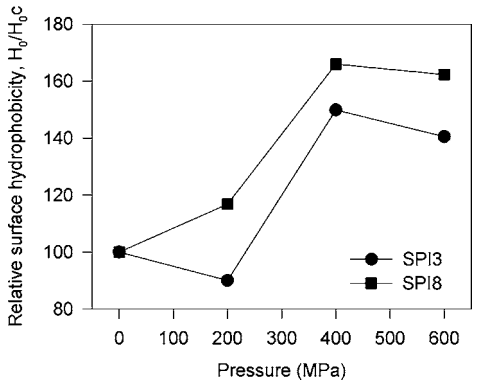

Figure 2. Surface hydrophobicity (H_0) of nontreated (0.1 MPa) and HP-treated (200–600 MPa) SPI dispersions (1% w/w). SPI dispersions: acidic-pH 3 (SPI3) and alkaline-pH 8 (SPI8).

Electrophoresis under Nondenaturing Conditions. Native electrophoresis of buffer-soluble extracts was performed in a 7% polyacrylamide running gel with a 3.5% polyacrylamide stacking gel. A continuous nondissociating buffer system containing 2 M Tris-base, pH 8.8, for the separating gel and 0.027 M Tris-base, 0.38 M glycine, pH 8.3, for the running buffer was used. The proteins were first stained with a Coomassie blue solution (Coomassie blue 0.05%, ethanol 25%, acetic acid 10%), and the gels were destained in a solution containing acetic acid (7%), ethanol (40%), and water (53%). The gels were scanned on an imaging densitometer Biorad GS710 (Biorad, Ivry-sur-Seine, France).

RESULTS AND DISCUSSION

Protein Solubility. Nontreated SPI presented higher solubility at pH 8 (SPI8) than at pH 3 (SPI3) (**Figure 1**). This behavior can be attributed to the known phenomenon of protein aggregation near the isoelectric point (pI). The solubility of SPI8 slightly changed as the HP treatment increased. For SPI3, the solubility increased over 200 MPa, while no differences were observed with higher pressures (400 and 600 MPa). Chapleau and de Lamballerie-Anton (*28*) obtained different results for the globular proteins of lupin. They found a decrease in lupin protein solubility at pressures higher than 200 MPa.

Surface Hydrophobicity. Figure 2 shows the variation of H_0 with HP. A significant increase in H_0 was observed from 400 MPa for both isolates (166 and 150% of the initial value for SPI8 and SPI3, respectively), indicating that pressure treatment produced a molecular unfolding of the protein with the exposure of the hydrophobic groups to the medium. The H_0 increase was more pronounced at alkaline pH. It has been suggested that pressure causes changes in the structure of protein molecules due to the cleavage of weak hydrogen bonds and van der Waals forces (*8*, *9*), probably with the exposure of hydrophobic groups. Molina et al. (*24*) also observed an increase in surface hydrophobicity of SPI, 7S, and 11S fractions at neutral pH with pressure treatment above 200 MPa. These authors attributed this behavior to partial denaturation and subsequent exposure of hydrophobic groups.

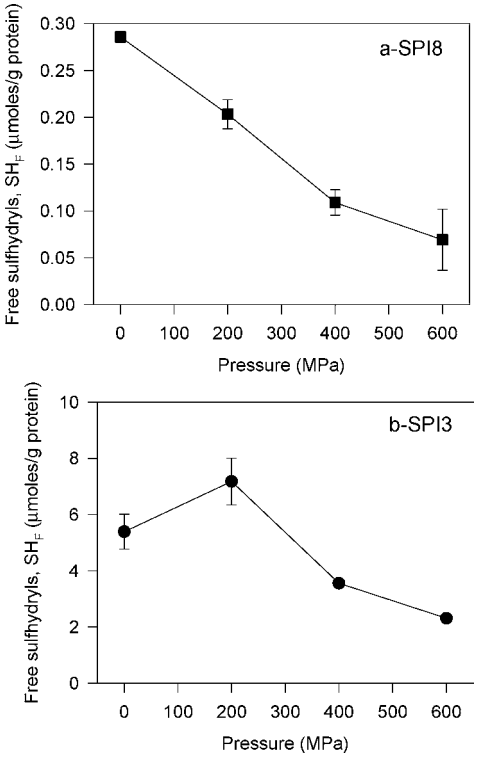

Figure 3. Free sulfhydryls (SH_F) of nontreated (0.1 MPa) and HP-treated (200–600 MPa) SPI dispersions (1% w/w). (**a**) alkaline dispersions (SPI8), (**b**) acidic dispersions (SPI3).

Free Sulfhydryls. Protein unfolding was accompanied by the formation of disulfides (S−S) induced by HP treatment (**Figure 3**). The content of SH_F of SPI8 decreased as the pressure increased (**Figure 3a**), while that of SPI3 began to decrease at pressures higher than 200 Mpa (**Figure 3b**). It was also observed that the content of free sulfhydryls groups was higher in the SPI3 than in the SPI8, due to the protonation of the groups at acidic pH. Formation of S−S bonds through SH/S−S interchange reactions would be accompanied by the formation of hydrophobic interactions. This phenomenon was previously observed in other pressure-treated proteins such as β-lactoglobulin by Funtenberger et al. (*30*, *31*).

Thermal Behavior. Thermograms of SPI8 are shown in **Figure 4**. The nontreated SPI8 sample presented the typical endotherms corresponding to the denaturation of the 7S and 11S fractions at 73.9 ± 0.53 °C and 86.7 ± 0.24 °C, respectively. Treatment at 400 and 600 MPa produced denaturation and thermal destabilization of both 7S and 11S fractions, with a new peak detected at 71 ± 0.13 °C. A reduction of the denaturation enthalpy of SPI of 73 and 79%, as compared to the nontreated sample, was observed at 400 and 600 MPa, respectively (**Table 1**).

To understand the modifications detected in the thermal behavior of the nontreated and HP-treated SPI8 isolate, the 7S and 11S fractions equally treated were analyzed. Nontreated 7S fraction showed a unique endotherm at 72.3 ± 0.44 °C (**Figure 4**) equivalent to the first peak of the SPI8 thermogram. At 200 MPa, a slight decrease in the peak cooperatively was

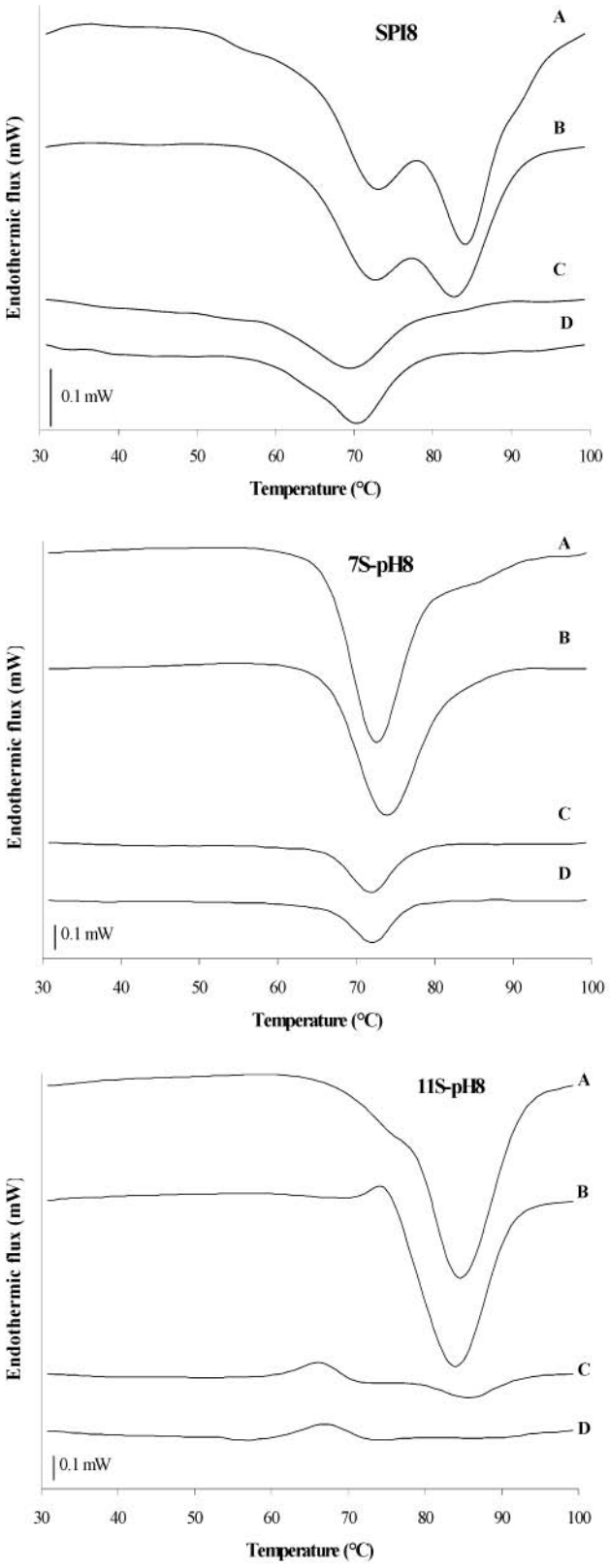

Figure 4. DSC thermograms of soybean proteins samples of pH 8: SPI, 7S, 11S. Pressure treatment: control (A), 200 MPa 10 min (B), 400 MPa 10 min (C), 600 MPa 10 min (D).

detected. No changes in thermal stability were detected with HP treatment. The enthalpies of pressurized samples at 200, 400, and 600 MPa were lower (11, 78, and 84%, respectively) than those of the corresponding control sample (**Table 1**). The nontreated 11S fraction also presented a unique endotherm at

Table 1. Effect of High Pressure Processing on Denaturation Enthalpy ΔH_T of Soybean Protein Isolate (SPI), 7S, and 11S Fractions at 5% in Buffer pH 8.0 (50 mM, Tris–HCl) and pH 3.0 (50 mM, Glycine–HCl)

treatment	ΔH_T (J/g)[d]		
	SPI8	7S fraction-pH8	11S fraction-pH8
control	9.56 ± 0.19^a	11.64 ± 0.48^a	12.64 ± 0.14^a
200 MPa 10 min	8.59 ± 0.65^b	8.62 ± 0.21^b	9.21 ± 0.05^b
400 MPa 10 min	2.57 ± 0.14^c	2.53 ± 0.15^c	1.68 ± 0.27^c
600 MPa 10 min	2.02 ± 0.03^c	1.96 ± 0.09^c	1.25 ± 0.07^c
	SPI3	7S fraction-pH3	11S fraction-pH3
control	3.73 ± 0.29^a	7.95 ± 0.18^a	2.85 ± 0.47^a
200 MPa 10 min	1.61 ± 0.02^b	5.09 ± 0.25^b	0.18 ± 0.03^b
400 MPa 10 min	0.00 ± 0.10^c	0.00 ± 0.08^c	0.00 ± 0.04^b
600 MPa 10 min	0.00 ± 0.12^c	0.00 ± 0.10^c	0.00 ± 0.03^b

[a–c] Means of ΔH_T at pH within a column with same superscripts are not significantly different ($P < 0.05$). [d] All values were means ± SD of three values.

84.8 ± 0.51 °C (**Figure 4**). The application of high pressure at 200 MPa did not affect denaturation temperature but decreased denaturation enthalpy (≈27%, **Table 1**). At 400 and 600 MPa a high decrease of the denaturation enthalpy was detected (≈90%, **Table 1**). For both samples, an exotherm at about 66 ± 0.11 °C was also observed, probably due to protein aggregation.

The DSC results revealed different effects of HP on the 7S and 11S fractions. The results suggest that HP treatments at 200, 400, and 600 MPa produced the unfolding of 7S and 11S fractions as well as the dissociation of glycinin. Molina et al. (*24*) suggested that pressure of 400 MPa dissociated the 7S fraction of SPI into partially or totally denatured monomers that enhanced surface activity, but the unfolding of the 11S polypeptides within the hexamer led to aggregation, negatively affecting the surface hydrophobicity of the SPI.

The nontreated SPI3 thermograms presented two endotherms (**Figure 5**). Denaturation enthalpy of this sample was also lower than that corresponding to the nontreated SPI8 dispersions (**Table 1**). These phenomena indicate that soybean proteins at acidic pH (pH 3) are more sensible to thermal treatments and are partially unfolded and dissociated (*18*, *32*, *33*). High-pressure treatment at 200 MPa produced an additional denaturation (57% compared to the nontreated SPI3 sample) without changes in the thermal stability of the proteins. Treatment at 400 and 600 MPa produced a total denaturation of the proteins (**Figure 5**).

The nontreated 7S fraction at pH 3.0 presented a unique endotherm at 63 ± 0.31 °C (**Figure 5**) and a denaturation enthalpy about 40% lower than that corresponding to the nontreated 7S fraction at pH 8.0. The HP treatment induced the unfolding of the protein, reaching complete denaturation at 400 MPa (**Table 1**).

The nontreated 11S fraction was destabilized and denatured at pH 3.0. The thermograms obtained showed a unique endotherm at 59 ± 0.23 °C with a 77% denaturation as compared to the nontreated 11S fraction at pH 8.0 (**Figure 5**). This endotherm is likely to correspond to the 7S form dissociated from 11S globulin that was previously observed at pH 3.0 by Puppo and Añón (*18*) and Lakemond et al. (*32*, *33*). At 200 MPa, the endotherm was shifted to lower temperatures (48 ± 0.09 °C), and the unfolding was around 94%. Higher pressures (400 and 600 MPa) produced total denaturation of glycinin. The comparison of the thermal behavior of the fractions with that of the SPI3 dispersion indicates that the first endotherm of the thermograms corresponds to the dissociated 11S fraction or

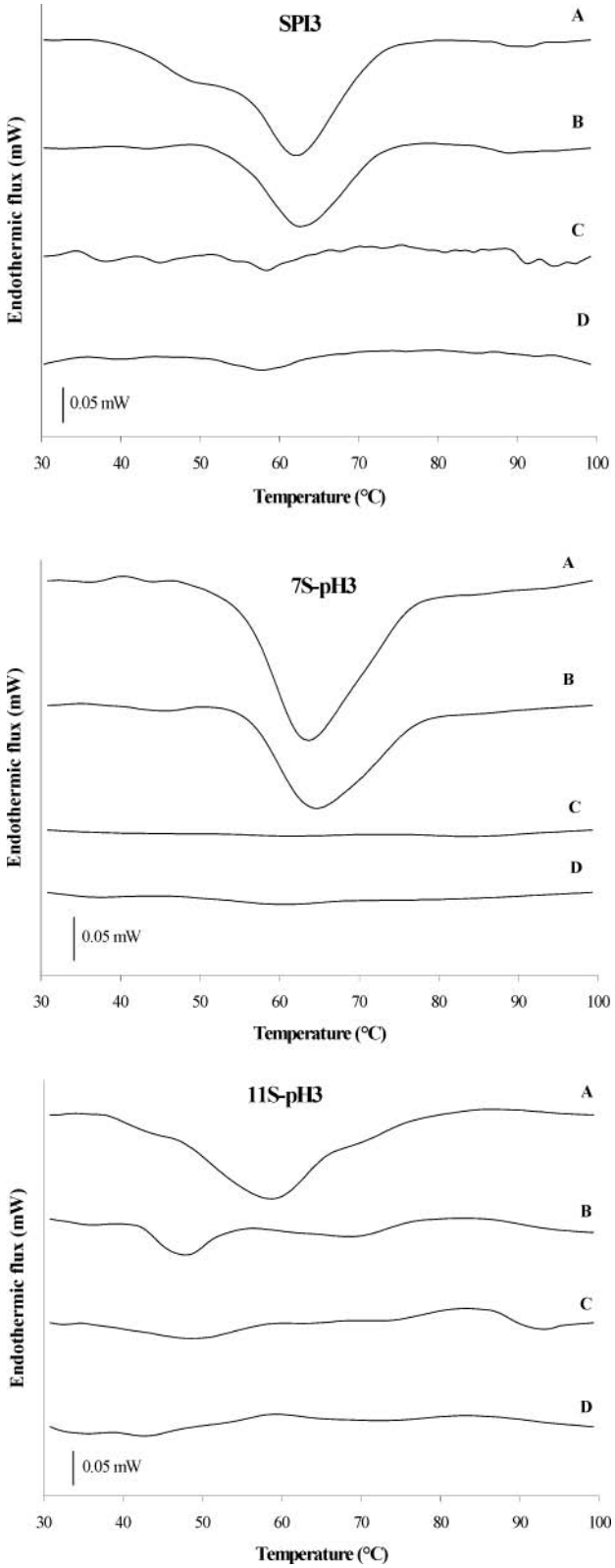

Figure 5. DSC thermograms of soybean proteins samples of pH 3: SPI, 7S, 11S. Pressure treatment: control (A), 200 MPa 10 min (B), 400 MPa 10 min (C), 600 MPa 10 min (D).

glycinin (7S form) and the second to the 7S fraction or β-conglycinin.

Secondary Structure Characterization. Circular dichroism spectra (far UV−CD) of the soluble fraction of SPI dispersions are shown in **Figure 6**. SPI proteins presented the typical spectra of $\alpha + \beta$ proteins described by Venyaminov and Yang (*36*).

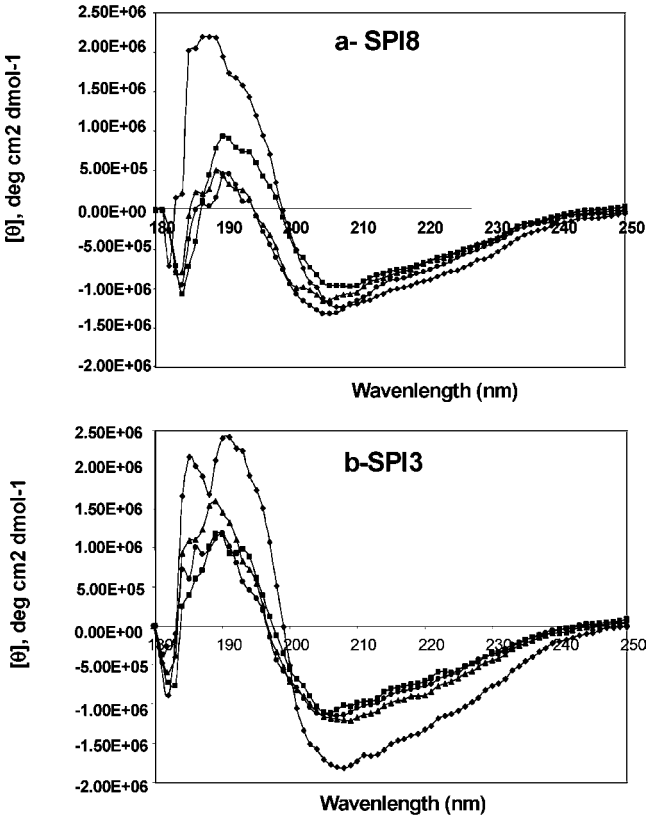

Figure 6. Circular dichroism spectra of SPI dispersions. (**a**) alkaline dispersions (SPI8), (**b**) acidic dispersions (SPI3). (◆) Control, (■) 200 MPa, (▲) 400 MPa, (●) 600 MPa.

Spectra presented a positive band near 190 nm with a zero crossing around 200 nm, and a negative band near 210 nm. Intensities of CD bands reflect the amount of ellipticity in the proteins (*34*). Secondary structures of nontreated SPI were slightly modified by the pH, as SPI3 presented about 37% α-helix, 13% β-sheets, 10% β-turns, and 40% random coils, while SPI8 presented about 40% α-helix, 12% β-sheets, 9% β-turns, and 39% random coils.

HP treatment induced changes in the secondary structure of SPI8 (**Figure 6a**). The intensity of the positive band decreased and the minimum of the negative band was shifted toward lower wavelength with HP treatment, specially at higher pressures (400 and 600 MPa). Lower zero-crossing values were also observed under these conditions. Nagano et al. (*14*) studied secondary structure changes in β-conglycinin (7S) with thermal treatment. They associated the shift of the negative peak toward 200 nm, due to the temperature increase, with conformational changes to a more disordered structure. We have calculated that the α-helix content decreased after 200 MPa treatment (24%), whereas the percentage of β-sheets increased concurrently (24%). For higher pressures (400 and 600 MPa), α-helix content dropped to about 15%, whereas β-sheets remained constant (23−24%). Random coil increased from 41% at 200 MPa to 48% at 400 and 600 MPa. Concerning the β-turn, their proportion increased up to 13% at 600 MPa.

SPI3 protein spectra are shown in **Figure 6b**. Changes in secondary structure with HP were observed, but the differences in CD spectra between the different pressures were less pronounced than those detected at pH 8. We calculated a decrease of the α-helix content (27, 24, and 22% for 200, 400, and 600 MPa, respectively). Concurrently, an increase of β-sheet content (19, 21, and 22%) and of the random coil content (44%,

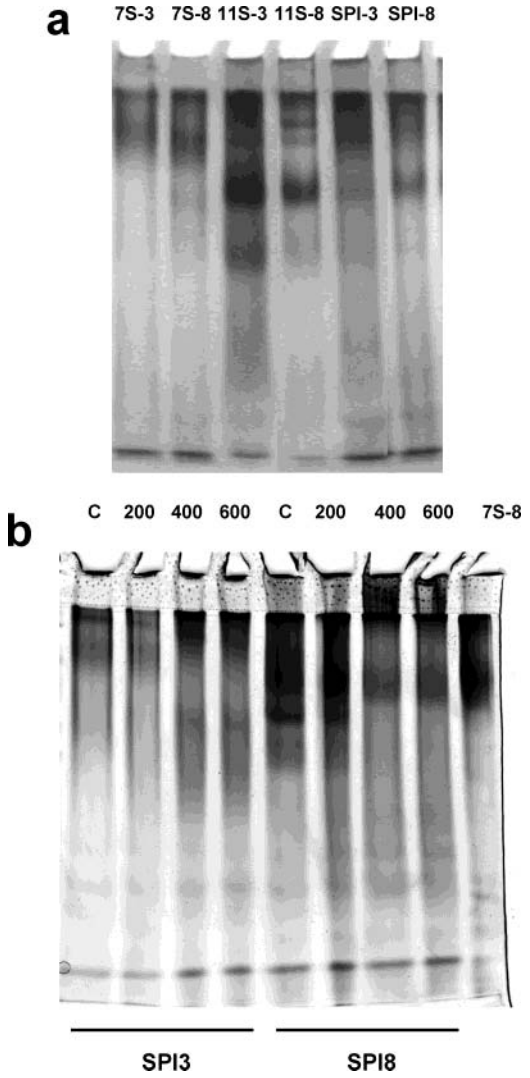

Figure 7. PAGE under nondenaturing conditions of SPI dispersions (1% w/w). Gel concentration: 7% of polyacrylamide. (**a**) Nontreated samples, (**b**) pressure-treated samples. HMW: molecular weight standard. C: control sample.

45% and 46%) was detected, whereas β-turns remained constant (10%). Other researchers (*8*, *9*) found a decrease in the α-helix content of proteins such as ovalbumin, lysozyme, BSA, and β-lactoglobulin at different pH conditions with HP treatment. The current study shows that, for SPI, the changes provoked by high-pressure treatments are more important at pH 8.

Aggregation Phenomenon. To study the effect of HP treatment on different protein species, nontreated acidic and alkaline (pH 3 and pH 8) samples of SPI and their fractions (7S and 11S) were analyzed by native electrophoresis (**Figure 7a**). The analysis of the two major subunits (7S and 11S) allowed to identify both fractions in SPI. Soluble SPI8 dispersion was composed by both 7S and 11S fractions (lanes SPI-8, 7S-8, and 11S-8). In contrast, soluble SPI3 was formed mainly by 7S, while 11S remained in the insoluble fraction (lanes SPI-3, 7S-3, and 11S-3). The high mobility protein species observed in 11S-3 would indicate the dissociation of that protein during pH treatment.

The nontreated SPI8 presented the common native electrophoretic profile with the presence of 7S and 11S bands (**Figure 7b**). Patterns remained unchanged at 200 MPa. HP processing (400 and 600 MPa) produced aggregation of the 11S fraction,

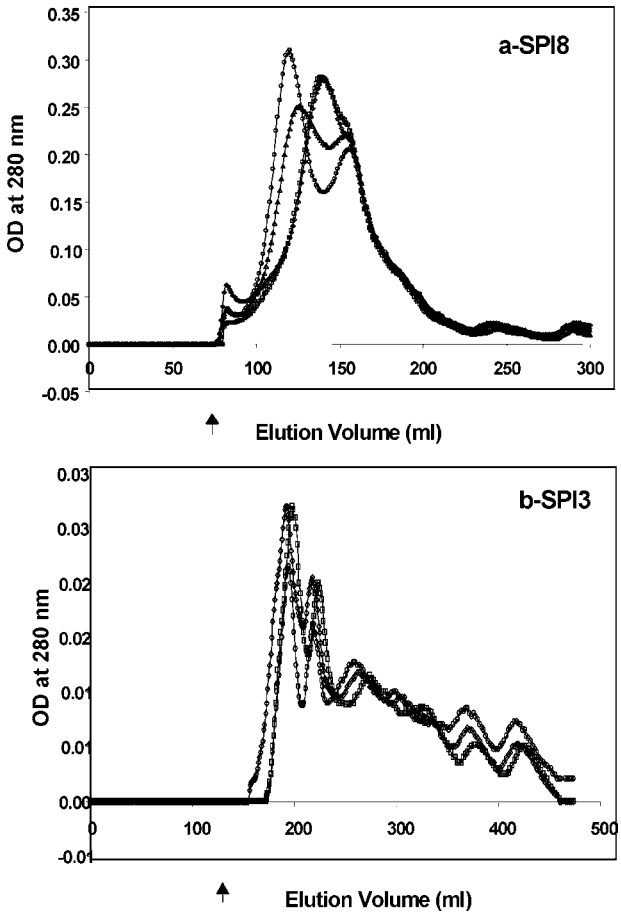

Figure 8. Size exclusion chromatograms of SPI dispersions. (**a**) alkaline dispersions (SPI8), (**b**) acidic dispersions (SPI3). Arrows indicate void volume position. (◇) Control, (□) 200 MPa, (△) 400 MPa, (○) 600 MPa.

yielding large aggregates that could not enter the gel, while no changes in 7S bands were observed.

As shown in **Figure 7a**, the soluble fraction of SPI3 did not present the typical bands corresponding to the 7S and 11S fractions observed at pH 8. Samples treated at 400 and 600 MPa showed a great amount of polypeptides of very low mobility. These findings can be attributed to the action of HP on protein aggregates present in the SPI3: partial depolymerization of insoluble aggregates by HP effect, followed by formation of new aggregates.

Size exclusion chromatography results of SPI dispersions are shown in **Figure 8**. Nontreated SPI8 presented a great peak of 813 kD, probably corresponding to native 7S and 11S subunits, with a shoulder (50 kD), and a second little peak of molecular mass higher than 1×10^6 kD (Figure 8a). Molina and Añón (*35*) also found a peak at 917 kD when native soybean protein isolate was analyzed by exclusion chromatography and suggested the presence of aggregated forms constituted by 7S and 11S fractions. No changes in chromatographic profiles were observed at 200 MPa. At 400 MPa, the first peak was split into two peaks and moved to low elution volumes (MM 11 343 kD). This process was more pronounced at the highest pressure (600 MPa, MM 29 618 kD). Chapleau and de Lamballerie-Anton (*28*) observed the same phenomenon with lupin proteins: The quaternary structure of 11S was more affected than that of 7S globulins by HP treatment (>400 MPa). Galazka et al. (*36*) previously described the same relationship between aggregation and SH/S−S interchange for neutral ovalbumin and 11S *Vicia faba* globulin. Funtenberger et al. (*30*, *31*) suggested that

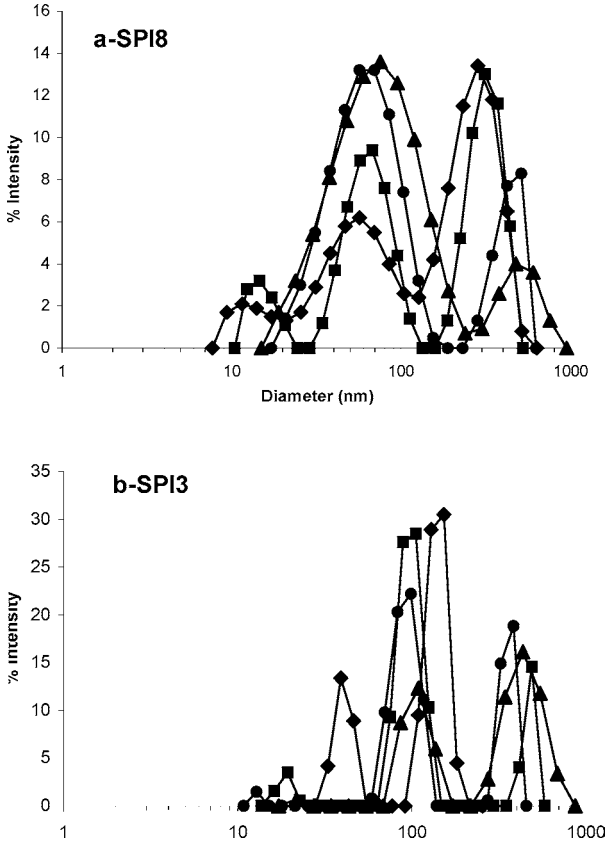

Figure 9. Quasi elastic light scattering (QELS). (**a**) alkaline dispersions (SPI8), (**b**) acidic dispersions (SPI3). Numbers on graphs indicate average diameter of particles of different populations. (◆) Control, (■) 200 MPa, (▲) 400 MPa, (●) 600 MPa.

formation of S−S bonds through SH/S−S interchange reactions would be also accompanied by the formation of hydrophobic interactions in pressured β-lactoglobulin at pH 7.

For the SPI3 (**Figure 8b**), two major peaks at elution volumes around 190 mL (I) and 220 mL (II), which corresponded to molecular masses lower than 66 kD, were observed for all samples. These results indicate that SPI3 contains a large amount of aggregates of high molecular weight that remain in the insoluble fraction, so that the soluble fraction only contains dissociated polypeptides. Some soluble aggregates would also be retained in the microfiltration step. The HP applied did not modify the molecular mass of polypeptides present in the soluble fraction. Results were in agreement with those obtained by native electrophoresis.

The average diameters of protein particles of nontreated or pressurized SPI dispersions are shown in **Figure 9**. For the nontreated SPI8 dispersions, individual protein molecules and two types of aggregates can be observed (**Figure 9a**). No changes in the average diameter distribution of proteins were observed at 200 MPa (**Figure 9a**). At 400 and 600 MPa, aggregation of proteins was detected (**Figure 9a**). These results were in agreement with those obtained by size exclusion chromatography.

In the case of the nontreated SPI3, two defined populations of proteins with different average diameters (40 and 139 nm) were observed (**Figure 9b**). The mean sizes detected indicate that proteins are aggregated, in agreement with the results of solubility and size exclusion chromatography studies. Pressure treatments at 200 to 600 MPa induced both the aggregation and deaggregation of proteins, with a predominance of the first process (**Figure 9b**).

The nontreated SPI8 is constituted by native β-conglycinin and glycinin, slightly aggregated but with high water solubility. Treatments at HP higher than 200 MPa produced important structural changes in the proteins: increase of the surface hydrophobicity, reduction of the SH_F and partial unfolding of the 7S and 11S fractions, change in secondary structure to a more disordered structure, and aggregation of the proteins, especially of the 11S fraction. The aggregates remained soluble and were stabilized by disulfide bridges and probably noncovalent bonds.

In the case of nontreated SPI3, proteins were partially denatured and presented insoluble aggregates. A decrease of thermal stability of proteins, particularly of the 11S fraction, was also detected. HP treatment produced an increase of protein solubility, mainly at 200 MPa. This change was attended by an increase of the surface hydrophobicity of the molecules and major unfolding. At 400 and 600 MPa, in addition to the increase in hydrophobicity, a decrease of the SH content and a total denaturation were also observed. According to the results obtained by quasi-elastic light scattering, the HP treatment induced both aggregation and dissociation of SPI3 proteins. These changes could interrelate with a change in the interfacial properties. This issue will be addressed in a future study.

LITERATURE CITED

(1) Gould, G. W. The microbe as a high-pressure target. In *High-Pressure Processing of Foods*; Ledward, D. A., Johnston, D. E., Earnshaw, R. G., Hasting, A. P. M., Eds.; Nothingham University Press: Loughborough, UK, 1995; pp 27−35.

(2) Galazka, V. B.; Ledward, D. A. Developments in high-pressure food processing. *Food Technol. Int. Eur.* **1995**, *12*, 123−125.

(3) Knorr, D. Process aspects of high-pressure treatment of food systems. In *Innovations in Food Processing*, Barbosa-Cánovas, G. V., Gould, G. W., Eds.; Technomic Publishing Company, Inc.: Lancaster, PA, 2000; Chapter 2, pp 13−30.

(4) Montero, P.; Fernández-Díaz, M. D.; Gómez-Guillén, M. C. Characterization of gelatin gels induced by high pressure. *Food Hydrocolloids* **2002**, *16*, 197−205.

(5) O'Reilly, C. E.; Kelly, A. L.; Murphy, P. M.; Beresford, T. P. High-pressure treatment: applications in cheese manufacture and ripening. *Trends Food Sci. Technol.* **2001**, *12*, 51−59.

(6) Silva, J. L.; Weber, G. Pressure stability of proteins. *Annu. Rev. Phys. Chem.* **1993**, *44*, 89−113.

(7) Balny, C.; Masson, P. Effects of high pressure on proteins. *Food Rev. Int.* **1993**, *9*, 611−628.

(8) Tedford, L. A.; Smith, D.; Schaschke, C. J. High-pressure processing effects on the molecular structure of ovalbumin, lysozyme, and β-lactoglobulin. *Food Res. Int.* **1999**, *32*, 101−106.

(9) Hayakawa, I.; Linko, Y.-Y.; Linko, P. Mechanism of high-pressure denaturation of proteins. *Lebensm.-Wiss. Technol.* **1996**, *29*, 756−762.

(10) Galazka, V. B.; Dickinson, E.; Ledward, D A. Emulsifying behavior of 11S globulin *Vicia faba* in mixtures with sulphated polysaccharides: comparison of thermal and high-pressure treatments. *Food Hydrocolloids* **1999a**, *13*, 425−435.

(11) Galazka, V. B.; Smith, D.; Ledward, D. A.; Dickinson, E. Interactions of ovalbumin with sulphated polysaccharides: effects of pH, ionic strength, heat and high-pressure treatment. *Food Hydrocolloids* **1999b**, *13*, 81−88.

(12) Yao, J. J.; Tanteeratarm, K.; Wei, L. S. Effects of maturation and storage on solubility, emulsion stability, and gelation properties of isolated soy proteins. *J. Am. Oil Chem. Soc.* **1990**, *67*, 974−989.

(13) Utsumi, S.; Kinsella, J. E. Structure−function relationships in food proteins: subunit interactions in heat-induced gelation of 7S, 11S, and soy isolate proteins. *J. Agric. Food Chem.* **1985**, *33*, 297−303.

(14) Nagano, T.; Akasaka, T.; Nishinari, K. Study of the heat-induced conformational changes of β-conglycinin by FTIR and CD analysis. *Food Hydrocolloids* 1995, *9*, 83−89.
(15) Petruccelli, S.; Añón, M. C. Relationship between the method of obtention and the structural and functional properties of soy protein isolates. 1. Structural and functional properties. *J. Agric. Food Chem.* **1994a**, *42*, 2161−2169.
(16) Petruccelli, S.; Añón, M. C. Relationship between the method of obtention and the structural and functional properties of soy protein isolates. 2. Surface properties. *J. Agric. Food Chem.* **1994b**, *42*, 2170−2176.
(17) Puppo, M. C.; Lupano, C. E.; Añón, M. C. Gelation of soybean protein isolates in acidic conditions. Effect of pH and protein concentration. *J. Agric. Food Chem.* **1995**, *43*, 2353−2361.
(18) Puppo, M. C.; Añón, M. C. Soybean protein dispersions at acidic pH. Thermal and rheological behavior. *J. Food Sci.* **1999**, *64*, 50−56.
(19) Cronakis, I. S. Network formation and viscoelastic properties of commercial soy protein dispersions: effect of heat treatment, pH, and calcium ions. *Food Res. Int.* **1996**, *29*, 123−134.
(20) Wagner, J. R.; Sorgentini, D. A.; Añón, M. C. Thermal and electrophoretic behavior, hydrophobicity, and some functional properties of acid-treated soy isolates. *J. Agric. Food Chem.* **1996**, *44*, 1881−1889.
(21) Sorgentini, D. A.; Wagner, J. R. Comparative study of foaming properties of whey and isolates soybean proteins. *Food Res. Int.* **2002**, *35*, 721−729.
(22) Yamauchi, F.; Yamagishi, T.; Iwabuchi, S. Molecular Understanding of Heat-Induced Phenomena of Soybean Protein. *Food Rev. Int.* **1991**, *7*, 283−322.
(23) Sorgentini, D. A.; Wagner, J. R.; Añón, M. C. Effects of thermal treatment of soy protein isolate on the characteristics and structure−function relationship of soluble and insoluble fractions. *J. Agric. Food Chem.* **1995**, *43*, 2471−2479.
(24) Molina, E.; Papadopoulou, A.; Ledward, D. A. Emulsifying properties of high-pressure-treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids* **2001**, *15*, 263−269.
(25) Molina, E.; Defaye, A. B.; Ledward, D. A. Soy protein pressure-induced gels. *Food Hydrocolloids* **2002**, *16*, 625−632.
(26) Nkonge, C.; Murray Ballance, G. A sensitive colorimetric procedure for nitrogen determination in micro-Kjeldahl digests. *J. Agric. Food Chem.* **1982**, *30*, 416−420.
(27) Nagano, T.; Hirotsuka, M.; Mori, H.; Kohyama, K.; Nishinari, K. Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. *J. Agric. Food Chem.* **1992**, *40*, 941−944.
(28) Chapleau, N.; de Lamballerie-Anton, M. Improvement of emulsifying properties of lupin proteins by high-pressure induced aggregation. *Food Hydrocolloids* **2003**, *17*, 273−280.
(29) Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe methods and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.
(30) Funtenberger, S.; Dumay, E.; Cheftel, J. C. Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. *Lebensm.-Wiss. Technol.* **1995**, *28*, 410−418.
(31) Funtenberger, S.; Dumay, E.; Cheftel, J. C. High-pressure promotes β-lactoglobulin aggregation through SH/S−S interchange reactions. *J. Agric. Food Chem.* **1997**, *45*, 912−921.
(32) Lakemond, C. M. M.; de Jongh, H. H. M.; Hessing, M.; Gruppen, H.; Voragen, A. G.J. Soy glycinin: Influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *J. Agric. Food Chem.* **2000a**, *48*, 1985−1990.
(33) Lakemond, C. M. M.; de Jongh, H. H. M.; Hessing, M.; Gruppen, H.; Voragen, A. G. J. Heat Denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *J. Agric. Food Chem.* **2000b**, *48*, 1991−1995.
(34) Venyaminov, S. Y.; Yang, J. T. Determination of protein secondary structure. In *Circular Dichroism and the Conformational Analysis of Biomolecules*; Fasman, G. D., Ed.; Plenum Press: New York, 1996; Chapter 3, pp 69−107.
(35) Molina, S. E.; Añón, M. C. Analysis of products, mechanisms of reaction, and some functional properties of soy protein hydrolysates. *J. Am. Oil. Chem. Soc.* **2000**, *77*, 1293−1301.
(36) Galazka, V. B.; Dickinson, E.; Ledward, D. A. Emulsifying properties of ovalbumin in mixtures with sulphated polysaccharides: effects of pH, ionic strength, heat, and high-pressure treatment. *J. Sci. Food Agric.* **2000**, *80*, 1219−1229.

Received for review July 22, 2003. Revised manuscript received October 28, 2003. Accepted January 29, 2004. This investigation was supported by the Bilateral Ecosud Project (A01B04) between France and Argentina.

JF034813T