Characterization of Soybean Protein Isolates. The Effect of Calcium Presence

A.A. Scilingo and M.C. Añón

Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), (1900) La Plata, Argentina

ABSTRACT: Results of a study of the effect of calcium addition on polypeptide composition, hydrophobicity, sulfhydryl content, thermal stability, enthalpy of denaturation, and water solubility of soybean protein isolates suggest that the addition of small amounts of calcium (1.23–5.0 mg/g protein) induced the formation of α,α′ soluble aggregates, whereas large amounts (5.0–9.73 mg/g protein) induced the selective insolubilization of the glycinin fraction. The addition of this ion also produced thermal stabilization of the soybean proteins, mainly the glycinin fraction, and increased the enthalpy of denaturation. A decrease in the surface hydrophobicity of proteins with increasing calcium content was also observed. The results obtained suggested the existence of specific calcium–soy protein interactions, especially with the glycinin fraction.

Paper no. J10341 in *JAOCS 81*, 63–69 (January 2004).

KEY WORDS: Calcium addition, solubility, soybean protein isolates.

New food ingredients with good-quality protein and low cost currently are being sought. Many of those being investigated include soy protein because soybean seeds contain a high amount of protein with good nutritional quality. Furthermore, glycinin (11S) and β-conglycinin (7S) globulins, the major components of soy protein isolates, possess appropriate functional properties for food applications. The structure and conformation of these proteins as well as substances present during the isolation procedure influence these properties.

Macromolecules are affected by salts. These effects will depend not only on salt concentration (ionic strength) but also on their nature (lyotropic effect). According to Hofmeister's series, which arranges ions according to the lyotropic effect they may have, the calcium ion is located at the end of this series, corresponding to those ions that promote protein salting-in, leading to destabilization of the native structure (1).

Calcium is an essential mineral, either by itself or when replacing sodium. It participates in the etiology or control of two widespread diseases: osteoporosis and arterial hypertension (2). Moreover, calcium intakes are often far below the recommended levels. Accordingly, its incorporation into nondairy foods is an appropriate method for achieving adequate intake.

*To whom correspondence should be addressed at Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), 47 y 116, (1900) La Plata, Argentina. E-mail: mca@biol.unlp.edu.ar

Although the interactions between soy proteins and calcium have been studied (3–7), no consensus has been reached. Although some researchers support the existence of specific interactions (4,7), others disagree (5).

The aims of this study were to obtain soy protein isolates enriched by calcium addition and to study the influence of this ion on some protein structural parameters and on the water solubility of the isolates.

MATERIALS AND METHODS

Preparation of isolates. Protein isolates were prepared from hexane-defatted soy flour (Bunge-Ceval, Brazil) containing 50% protein (NSI 78-80). The flour was extracted with water adjusted to pH 8.0 with 2 N NaOH (flour/water ratio of 1:10 wt/vol) at room temperature for 2 h. The pH was periodically readjusted to 8.0. This suspension was filtered through gauze, and the filtered material was centrifuged at 10,000 × *g* at 4°C for 30 min. The supernatant was adjusted to pH 4.5 with 2 N HCl. The precipitate was separated by centrifugation at 5,000 × *g* at 4°C for 15 min and then suspended in water. The suspension, which contained 7% (wt/vol) of proteins, was divided into aliquots, which were adjusted to pH 8.0 with 2 N NaOH (control proteinate) or NaOH plus 5.5 mg/mL $Ca(OH)_2$. Different amounts of calcium were added to the aliquots during suspension. The calcium content of the soybean protein isolates ranged from 1.23 to 9.73 mg/g protein (Table 1). The suspensions were freeze-dried and stored at 4°C.

At least two preparations of each calcium isolate were performed.

Protein determination. The protein content of isolates was measured by the Kjeldahl method ($f = 5.7$) (8). Protein concentration was determined using either the biuret method (9) or the method of Lowry *et al.* (10) as an alternative for diluted solutions.

At least two determinations were carried out for each sample.

Solubility in water. Soybean protein isolates were suspended in water (5% wt/vol) at room temperature for 1 h with continuous shaking and then centrifuged at 10,000 × *g* at 4°C for 10 min. The solubility was expressed as the percentage (w/w) of the soluble fraction compared to the starting sample. At least two determinations were carried out for each sample.

SDS-PAGE electrophoresis. (i) Unidimensional SDS-PAGE. Dissociating electrophoresis was carried out in a continuous buffer system: 0.375 M Tris-HCl, pH 8.8, 0.1% (wt/vol) SDS

Copyright © 2004 by AOCS Press

for the separating gel. Sample buffer used was 0.125 M Tris-HCl, pH 6.8, 20% (vol/vol) glycerol, 1% (wt/vol) SDS, and 0.05% (wt/vol) bromophenol blue. In some cases, 5% (vol/vol) β-mercaptoethanol was added. Samples were prepared by mixing an aliquot of the soluble fraction with sample buffer. The buffer used for the electrophoretic runs was 0.025 M Tris-HCl, 0.192 M glycine, and 0.1% (wt/vol) SDS, pH 8.3. The gel was prepared with a 5–15% acrylamide gradient. Runs were performed at 20 mA/0.75 mm plate thickness and a maximum voltage of 200 V. Gels were fixed, stained with R-250 Coomassie (0.1% wt/vol) in water/methanol/acetic acid (5:5:2) overnight, and destained with 25% (vol/vol) methanol and 10% (vol/vol) acetic acid.

Densitograms were obtained by means of a TLC Scanning CS-910 double-wavelength Shimadzu spectrodensitometer.

Molecular weights of the proteins were estimated by means of the LMW Sigma kit (bovine albumin, 66.0; ovalbumin, 45.0; glyceraldehyde-3-phosphate dehydrogenase, 36.0; carbonic anhydrase, 29.0; trypsinogen, 24.0; trypsin inhibitor, 20.1; and α-lactalbumin, 14.2) or the LMW Pharmacia kit (phosphorylase B, 94.0; albumin bovine, 67.0; ovoalbumin, 43.0; carbonic anhydrase, 30.0; trypsin inhibitor, 20.1; and α-lactalbumin, 14.4).

Bidimensional gel electrophoresis SDS-PAGE and SDS-PAGE + 2-mercaptoethanol (2-ME). Each lane of the first-dimension slab gel was treated with 10 vol of treatment buffer [62.5 mM Tris-HCl pH 6.8, 1% (wt/vol) SDS, sucrose 20% (wt/vol), and 2-ME (0.2 M)] at 55°C for 30 min. The procedure was repeated twice, changing the treatment buffer. Immediately, this portion of the gel was placed on top of the gel used for the second dimension. Runs were performed under the same conditions mentioned for the unidimensional gel.

DSC. A DuPont 910 DSC device was used for these studies. The cell constant and temperature calibrations were performed according to ASTM Norms E 967-83 (11) and E 968-83 (12) using indium thermograms, respectively. Hermetically sealed aluminum pans that contained 13–20 mg of soybean protein isolate suspended in water (20% wt/vol) were prepared. These capsules were scanned at 10°C/min over the range 30–130°C. An empty double pan was used as reference. After DSC analysis, the pans were punctured and the dry matter weight was determined by drying at 105°C overnight. At least duplicate samples were taken. The parameters derived from the thermograms were the denaturation temperature (T_d) and denaturation enthalpy (ΔH_T) of soy globulins. A cooperativity degree of the thermal transition of glycinin was also determined from the width at half-peak-height ($\Delta T_{1/2}$).

Determination of free sulfhydryl (SH_F) groups. SH_F groups were determined according to the procedure of Beveridge *et al.* (13) by dissolving 50 mg of isolate in 5 mL of a buffer containing 0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, and 8 M urea, pH 8.0. Forty milliliters of Ellman's reagent (4 mg/mL in methanol) was added to 1 mL aliquots. Absorbance at 412 nm was determined at different times until the absorbance maximum was reached. A molar extinction coefficient of 13,600 M^{-1} cm^{-1} was used. Protein concentration was determined according to the biuret method (9) using bovine albumin dissolved in 8 M urea for plotting the calibration curve. Duplicate determinations were made. The number of SH_F was calculated according to the following equation:

$$SH_F = 73.53\ \frac{(DO_1 - DO_2 - DO_3)}{C} \qquad [1]$$

where SH_F is concentration of free sulfhydryl in the sample (μmol/g protein); DO_1, maximum absorbance at 412 nm of sample + Ellman's reagent; DO_2, absorbance at 412 nm of sample without Ellman's reagent; DO_3, absorbance of Ellman's reagent at 412 nm in urea buffer at 8 M; C, sample protein concentration (mg/mL). The number 73.53 is a coefficient that includes ε (13,600 M^{-1} cm^{-1}) and constants to express sulfhydryl content in μmol/g protein.

Determination of surface hydrophobicity (S_O). Values of S_O were determined by using the hydrophobicity fluorescence probe 1-anilino-8-naphthalene sulfonate (ANS) according to the method described by Hayakawa and Nakai (14). Fluorescence intensity (FI) was measured at 365 nm (excitation) and 484 nm (emission) using a PerkinElmer 2000 fluorometer. The initial slope of the FI vs. protein concentration plot (calculated by linear regression analysis) was used as an index of protein hydrophobicity (S_O). Protein concentration was determined by the method of Lowry *et al.* (10). At least two determinations were performed for each sample.

Statistical analysis. The data obtained were statistically judged by ANOVA. The comparison of means was done by the Tukey test at a level (P) of 0.05. Both tests were carried out using the statistical analysis package SYSTAT (15).

RESULTS AND DISCUSSION

Water solubility. Water solubility of the soy protein isolates (percentage of bulk soluble protein/total sample) is presented in Figure 1. The sodium proteinate used as control showed a high solubility in water (about 80%). Calcium addition up to 5 mg ion/g isolate protein did not significantly affect solubility. From this calcium content upward, solubility decreased with calcium increase.

Electrophoretic analysis. The polypeptidic composition of the isolate soluble fraction was judged by SDS-PAGE (Fig. 2) to determine which proteins were involved in the decrease of solubility. Control sodium proteinate (Fig. 2A) contained those subunits corresponding to β-conglycinin (α′, α, and β), AB-glycinin subunit, and a small amount of their free A and B polypeptides. Soluble aggregates were observed in the high M.W. region; some of them were unable to penetrate the polyacrylamide gel (named 1) whereas others did (named 2 and 4, 113 ± 3 and 86 ± 2 kDa, respectively). A strong band of 28.5 ± 0.7 kDa, named 3, also was detected. The soluble protein fraction of the isolates after calcium addition evidenced the same polypeptide composition when compared to the control. However, the relative intensities of bands showed marked differences (Figs. 2B–F). All the proteinates maintaining water solubility similar to the control showed identical electrophoretic profiles (Figs. 2B–D). The region corresponding to the aggregates that penetrate the polyacrylamide gel appeared to be increased, whereas the bands of β-conglycinin

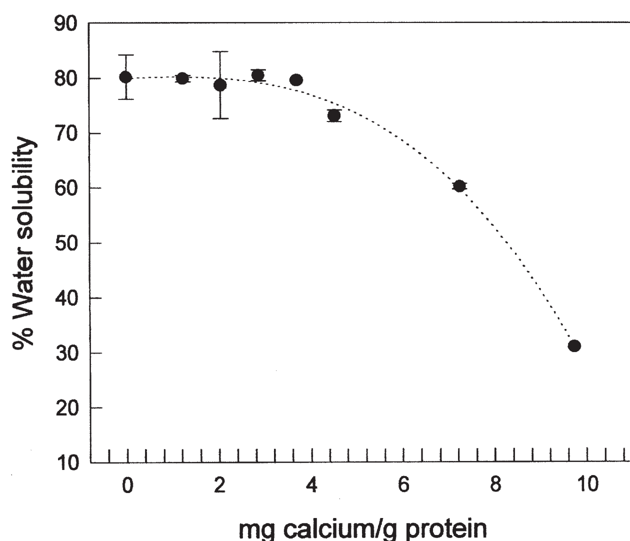

FIG. 1. Percent solubility in water of soybean protein isolates as a function of their calcium content.

α′, α, and β subunits showed a diminished relative intensity compared to that of the control (Figs. 2B–D vs. A). Densitograms of the isolates with high calcium content and lower water solubility than the control (Figs. 2E, F) provided good evidence regarding the predominance of β-conglycinin bands in the soluble fraction, although the intensity of the AB-subunit of glycinin was found to be markedly decreased. These results indicate that, when the addition of calcium is insufficient to produce changes in water solubility, β-conglycinin is involved mainly in the formation of soluble aggregates. On the contrary, glycinin is responsible for the formation of soluble aggregates under precipitation conditions. Moreover, the 28.5 ± 0.7 kDa polypeptide soluble fraction is selectively enriched, becoming the only predominant protein species in the soluble fraction of the isolate with maximal calcium content (Fig. 2F).

The addition of 2-ME to the soluble fraction of these isolates led to notable changes in the electrophoretic profiles. Figure 3 shows the control isolate (Fig. 3A), a soy isolate with low calcium content and high water solubility (Fig. 3B), and another one with high calcium content and low water solubility (Fig. 3C). We found that those soluble aggregates of high M.W. that penetrated the gel (named 2 and 4, Fig. 2) were not present in the control isolate (Fig. 3A) or in the low- and high-calcium content isolates (Figs. 3B and 3C, respectively). This fact revealed the involvement of disulfide bonds in the formation and stabilization of the aggregates. Also, the 28.5 ± 0.7 kDa protein band (band 3 in Fig. 2) was split owing to the action of the disulfide bond-reducing agent. The AB-glycinin subunit also disappeared, since its polypeptidic components are linked by disulfide bonds. The aggregates named 1 were partially separated, indicating that they may be stabilized by other interactions in addition to the covalent ones; the noncovalent interactions should hinder the 2-ME action. The comparison between the control isolate (Fig. 3A) and isolates modified by calcium addition (Figs. 3B,C) allowed us to detect a significant increase in the intensity of bands

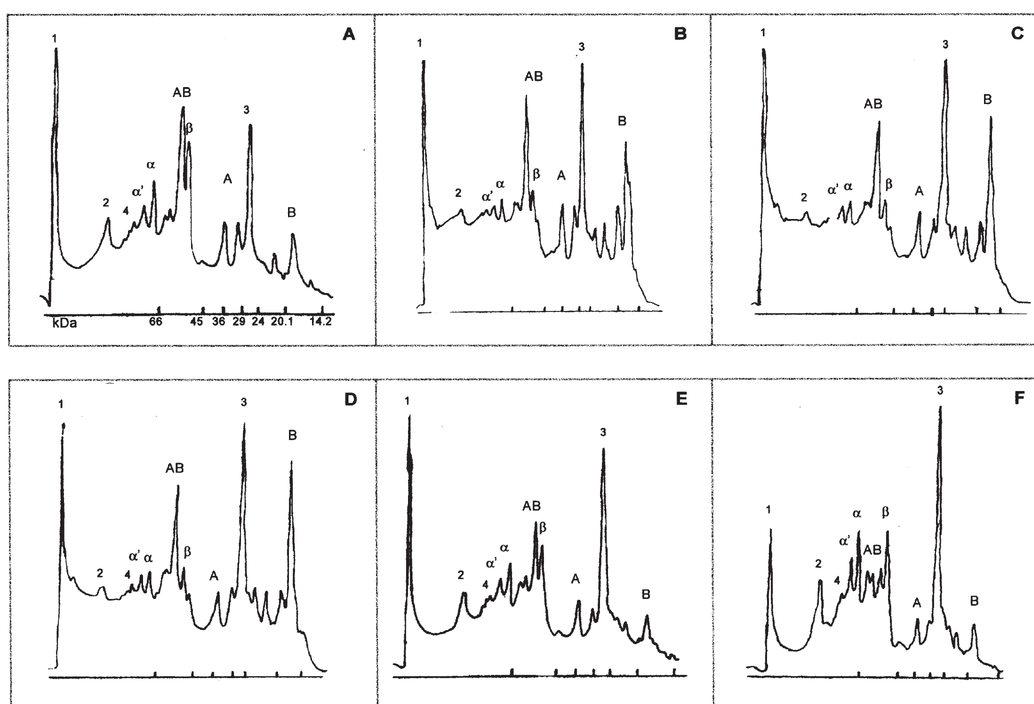

FIG. 2. Densitometric scans of the electrophoretic patterns (SDS-PAGE) of water-soluble fractions. (A) Sodium isolate (sample 1); (B–F) isolates modified by addition of 1.23, 2.87, 4.52, 7.24, and 9.73 mg calcium/g protein, respectively (samples 2, 4, 6–8). α′, α, β, conglycinin subunits: AB, glycinin subunits; A and B, glycinin polypeptides; 1, 2, and 4, soluble aggregates; and 3, polypeptide bands of 28 kDa approximately.

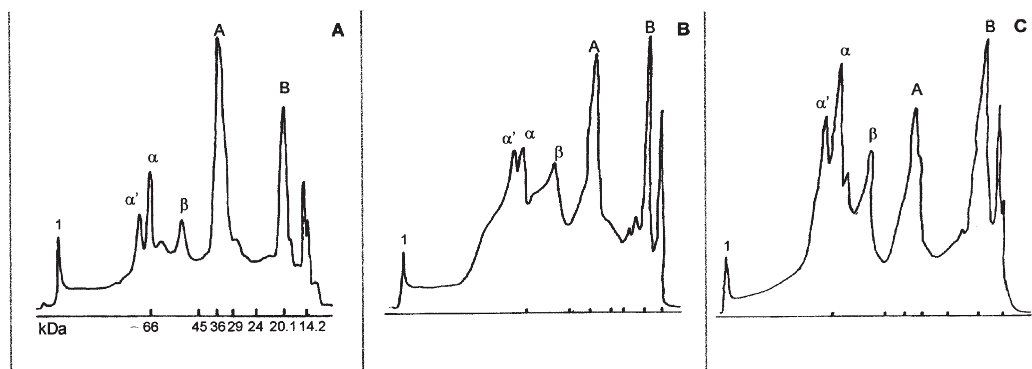

FIG. 3. Densitometric scans of the electrophoretic patterns [SDS-PAGE with 2-mercaptoethanol (2-ME)] of water-soluble fractions. (A) Sodium isolate (sample 1); (B and C) isolates modified by addition of 4.52 and 9.73 mg calcium/g protein, respectively (samples 6 and 8). For abbreviations see Figure 2.

corresponding to β-conglycinin (α′, α, and β), as well as a relative decrease in the intensity of bands corresponding to the AB-glycinin polypeptides; the higher the calcium content, the greater the change in the relative intensity of these bands. The increased intensity of the β-conglycinin polypeptides could be the result of the splitting of aggregates 2 and 4, produced by the reducing action of 2-ME. Also, the band named 3 was probably separated into a 14.8 ± 0.4 kDa polypeptide since it is the only new band in the profiles, and it is most likely linked to the increased intensity of the B-glycinin polypeptide (18.8 ± 0.5 kDa).

The polypeptidic composition of the soluble fractions in 8 M urea also was determined by SDS-PAGE. Densitograms of the isolates (soluble fractions at 8 M urea) were similar, irrespective of the presence and amount of calcium (Fig. 4). In the presence of 8 M urea, the aforementioned aggregates were detected (1, 2, and 4), indicating that covalent interactions are involved in their stabilization. A large amount of the β subunit of β-conglycinin, and of A- and B-glycinin polypeptides was observed in the 8 M urea profiles, as compared with those of the same isolates after their solubilization in water (Figs. 4A–C and Figs. 2A, D, F, respectively). In an aqueous medium these polypeptides induce aggregates stabilized by hydrophobic interactions and hydrogen bonds, which are disrupted in 8 M urea. A greater relative intensity also was found in the aggregate 2 band. These results allowed us to determine which proteins were either totally (β subunit of β-conglycinin, and A- and B-glycinin) or partially (band 2) disaggregated due to the effect of 8 M urea, forming bands consistent with individual subunits or high molecular mass aggregates as determined by SDS-PAGE. Bidimensional electrophoresis (Fig. 5) was performed to corroborate the covalent nature of those aggregates found in water and 8 M urea soluble fractions. Isolate 8 (9.73 mg calcium/g protein) was selected due to the fact that the presence of calcium increases the formation of aggregates. The soluble aggregates, unable to penetrate the polyacrylamide gel, were composed of the α and α′ subunits of β-conglycinin and the A- and B-glycinin polypeptides, whereas aggregates 2 and 4 were probably constituted by α and α′. The glycinin polypeptides might also be involved, although in a minor proportion. In contrast, the β subunit would not take part in the formation

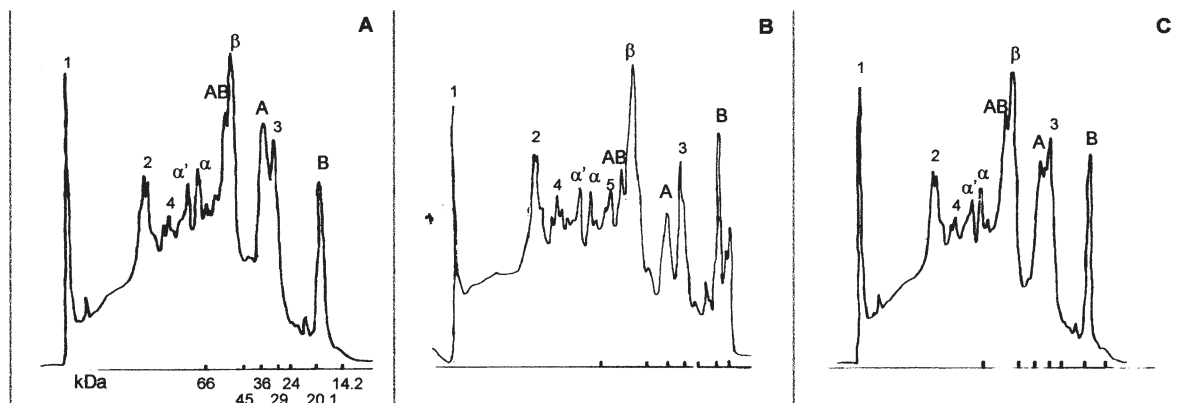

FIG. 4. Densitometric scans of the electrophoretic patterns (SDS-PAGE) of 8 M urea soluble fractions. (A) Sodium isolate (sample 1); (B and C) isolates modified by addition of 4.52 and 9.73 mg calcium/g protein, respectively (samples 6 and 8). For abbreviations see Figure 2.

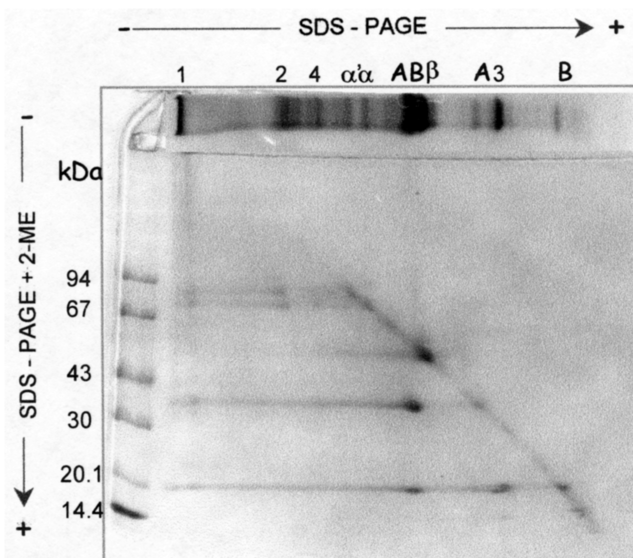

FIG. 5. Bidimensional electrophoresis (SDS-PAGE and SDS-PAGE + 2-ME) of 8 M urea-soluble fractions of sample 8 (9.73 mg calcium/g protein). For abbreviations see Figures 2 and 3.

of these aggregates. It is also evident that band 3 (28.5 ± 0.7 kDa) is split by 2-ME, giving rise to a polypeptide equal to β-glycinin, and a smaller polypeptide that was present in a lower quantity. This protein (band 3) might correspond to A_5B_3, that is, the disulfide-bound fraction of glycinin, $A_4A_5B_3$ globulin (also named G_4) (16).

Hoshi and Yamauchi (17) determined by electrophoresis a band with features similar to band 3 regarding molecular mass and modification by 2-ME. These authors also attributed its occurrence to B-glycinin dimers. Notwithstanding, as the molecular mass of B_2 does not reach 29 kDa, we support the hypothesis dealing with G_4, which may represent up to 10% of total glycinin in some soy variants (16).

Thermal analysis. Samples were studied by DSC (18). The T_d obtained showed that the glycinin structure is more stabilized than that of β-conglycinin as a result of the presence of calcium. This behavior was unexpected since calcium is located at the end of Hofmeister's series, with ions tending to produce an unstable protein structure owing to the decrease of hydrophobic interactions.

Thermal stabilization of some proteins due to increase of ion strength (19) should be discarded in our system because it remained almost constant in all the isolates assayed. In addition, the effects on globulins were different and selective by each globulin fraction.

ΔH values were similar for all soybean protein calcium isolates (Table 1) except for samples 2 and 3, which showed higher values ($P < 0.05$). In some cases, specific ion–protein binding (20) produces an increase of the denaturing ΔH due to the presence of the ion. This phenomenon would explain the ΔH increase in some calcium-containing isolates (samples 2 and 3). However, higher calcium contents (from 2.87 mg/g protein) would give rise to aggregation due to the action of both the presence of calcium and heating throughout the calorimetric run. The ΔH decrease observed could be due to the exothermal aggregation process and/or the disruption of the hydrophobic interactions that contribute to the stabilization of the aggregates promoted by the ion.

Calcium content increased the denaturing process cooperativity as revealed by the reduction of the $\Delta T_{1/2}$ value (Table 1) in those isolates with the highest calcium levels. This result suggests that soybean proteins in the presence of high calcium content may aggregate to form compact structures with higher cooperativity; a similar behavior was detected in preheated vegetable proteins (21).

Free sulfhydryl groups. Sulfhydryl groups (SH) and disulfide (SS) bonds are important in protein conformation since they give the macromolecule structural rigidity and stability. The soybean globulins, β-conglycinin and glycinin, contain different proportions of cysteine residues in their sequence, glycinin being the richest fraction (38–42 residues/mol) (22). The SH_F contents of the isolates under study are presented in Figure 6. All the values were low, and within a relatively narrow range (approximately 0.5–0.8 μmol SH_F/g protein). The SH_F values were lower than those reported by Wolf (16) considering the number of cysteines in the protein fraction. The decrease in the SH_F content could be attributed to the natural tendency to form SS bonds in the proteins (17), and also to the

TABLE 1
Temperatures (T_d), and Enthalpies (ΔH_T) of Denaturation of Soybean Globulins, and of Cooperativity Degree of Glycinin ($\Delta T_{1/2}$)

Soybean isolates	mg of calcium/g of protein	mmol of calcium/g of protein	T_d (°C)[a] β-Conglycinin	T_d (°C)[a] Glycinin	$\Delta T_{1/2}$ glycinin (°C)[a]	ΔH_T (J/g)[a]
1	0	0	75.5[a]	90.9[a]	9.50[a]	15.00[a,c]
2	1.23	0.03	77.0[b]	93.5[b]	9.50[a]	18.48[b]
3	2.05	0.05	73.5[c]	92.5[b]	9.50[a]	17.81[b]
4	2.87	0.07	74.0[c]	93.6[b]	9.02[b]	14.63[a,c]
5	3.70	0.09	74.1[c]	93.6[b]	9.50[a]	16.22[a]
6	4.52	0.11	76.0[a,b]	94.7[b,c]	9.50[a]	14.71[a,c]
7	7.24	0.18	76.9[b]	95.8[c]	8.50[c]	ND[b]
8	9.73	0.24	78.6[d]	97.3[d]	8.00[d]	14.03[a,c]

[a]Means in the same column with different superscript roman letters are significantly different ($P \leq 0.05$).
[b]ND, not determined.

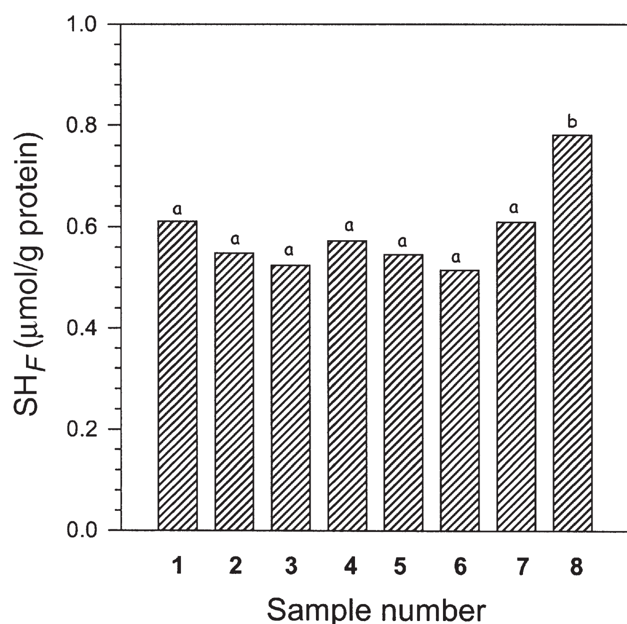

FIG. 6. Free sulfhydryl groups (SH_F) of the isolates studied. Samples 1 to 8: 0, 1.23, 2.05, 2.87, 3.70, 4.52, 7.24, and 9.73 mg calcium per g protein. Bars with different letters on the top are significantly different at $P \leq 0.05$.

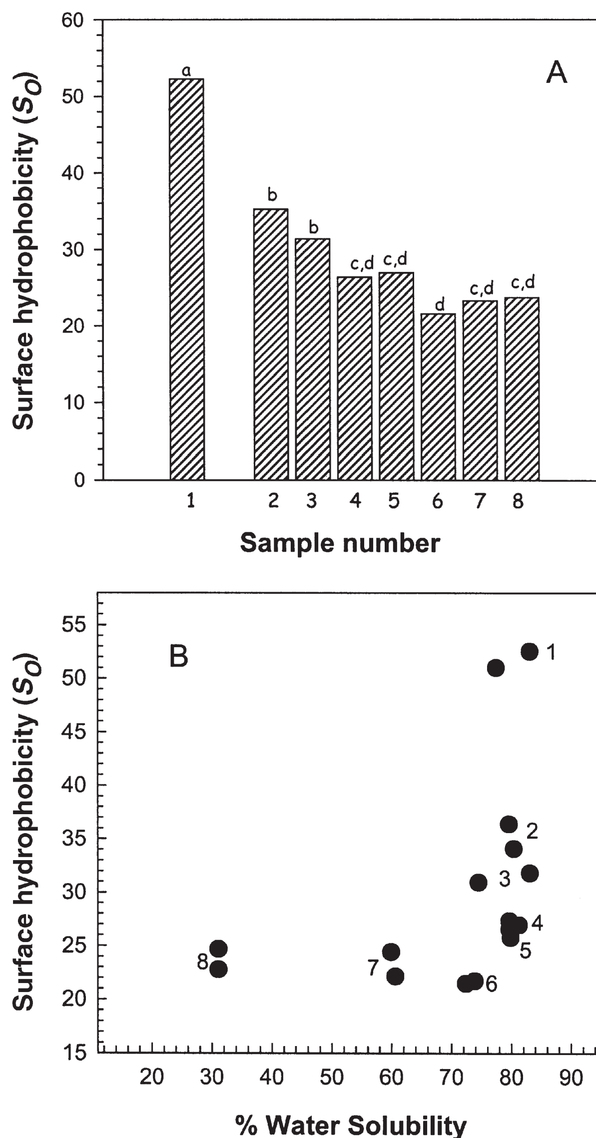

FIG. 7. (A) Values of surface hydrophobicity (S_O) determined by the interaction of the fluorescent probe 1-anilino-8-naphthalene sulfonate with the isolates studied. Bars with different letters on the top are significantly different at $P \leq 0.05$. (B) Relationship between S_O and water solubility (%). Samples 1 to 8: 0, 1.23, 2.05, 2.87, 3.70, 4.52, 7.24, and 9.73 mg calcium per g protein.

processes, i.e., lyophilization, to which the proteins are subjected (16,23).

Our results indicate that, except at the maximal calcium concentration, the isolates had the same SH_F content as sodium proteinate (samples 1 to 7, $P \leq 0.05$). After the addition of 9.73 mg ion/g protein (sample 8), the content of sulfhydryls was significantly different from and greater than in the other samples (0.76 ± 0.06 µmol SH_F/g protein, $P \leq 0.05$). Therefore, the presence of calcium would exert a masking effect on the reactive groups, partially avoiding the SS formation.

Surface hydrophobicity. S_O was assessed with ANS as fluorescent probe. Since changes of solubility are important for the samples under study, it should be noted that measurements were performed in the soluble fraction, in 0.1 M phosphate buffer at pH 7.0. Analysis of the calcium-containing isolates (Fig. 7A) indicated that their S_O were smaller than the ones of the soy protein isolate without calcium ($P \leq 0.05$). Also, S_O decreased as calcium content increased. Isolates 4–8 showed the same S_O, although less than that found at lower calcium content ($P \leq 0.05$).

Soy protein aggregation promotes a reduction in S_O (24), although there is no general rule for predicting hydrophobicity variations with changes in protein conformation. Therefore, the explanation for those changes in S_O must be the formation of soluble aggregates promoted by the presence of calcium. Such aggregates, with fewer regions within the reach of the probe than the control sodium proteinate (Fig. 7A), are soluble, since the S_O of soluble proteins has been quantified. Results previously obtained in our laboratory (25) showed that the presence of calcium does not prevent the probe interaction with the proteins.

The fluorescent probe could have had difficulty reaching the hydrophobic sites because they are closely related to the aggregate formation through hydrophobic bonds or, more likely, because the calcium ion could mask those sites by the ionic linkage of the polypeptidic chains; alternatively, both mechanisms could be involved.

Figure 7B shows the correlation between S_O and water solubility of the isolates studied. When enough calcium is added so as to induce solubility changes (≥4.52 mg calcium/g protein), the surface hydrophobicity remains unaltered. On the other hand, when water solubility remains stable, S_O increases. The present results indicate that calcium promotes the formation of aggregates that are initially soluble, in part because they

are stabilized by hydrophobic interactions. After calcium addition, their interaction with the fluorescent probe falls. Then the increase in size renders the aggregates insoluble, leading to precipitation, and the S_O of the soluble fraction remains constant.

Although the use of calcium salts as precipitating agents of soybean proteins dates back as far as two millenia, the nature of the ion–protein interaction still awaits clarification. Our results denote the existence of a specific calcium–soy protein interaction. On the other hand, the calcium soybean protein isolates obtained would be a good option for incorporating this mineral into nondairy foods.

ACKNOWLEDGMENTS

This investigation was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and by grants from Banco Interamericano de Desarrollo-Secretaría de Ciencia, Tecnología e Innovación Productiva. We also wish to thank Aldo H. Campana for his devotion and excellent technical assistance.

REFERENCES

1. Damodaran, S., and J.E. Kinsella, Effects of Ions on Protein Conformation, in *Food Protein Deterioration*, edited by J. Cherry, American Chemical Society, Washington, DC, 1982, pp. 301–357.
2. Suárez, F.L., and D.A. Savaiano, Diet, Genetics, and Lactose Intolerance, *Food Technol. 51*:74–76 (1997).
3. Appu Rao, A.G., and M.S. Narasinga Rao, Binding of Ca(II), Mg(II), and Zn(II) by 7S Fraction of Soybean Proteins, *J. Agric. Food Chem. 24*:490–494 (1976).
4. Appu Rao, A.G., and M.S. Narasinga Rao, Binding of Ca(II) by the 11S Fraction of Soybean Proteins, *Cereal Chem. 52*:21–33 (1975).
5. Sakakibara, K., and H. Noguchi, Interaction of 11S Fraction of Soybean Protein with Calcium Ion, *Agric. Biol. Chem. 41*:1575–1580 (1977).
6. Tezuka, M., H. Taira, Y. Igarashi, K. Yagasaki and T. Ono, Properties of Tofus and Soy Milks Prepared from Soybeans Having Different Subunits of Glycinin, *J. Agric. Food Chem. 48*:1111–1117 (2000).
7. Kroll, R.D., Effect of pH on the Binding of Calcium Ions by Soybean Proteins, *Cereal Chem. 61*:490–495 (1984).
8. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 14th edn., edited by S. Williams, Association of Official Analytical Chemists, Arlington, VA, 1984, Method 2.057, p. 16.
9. Gornall, A.C., C.J. Bardawill, and N.M. David, Determination of Serum Proteins by Means of the Biuret Reaction, *J. Biol. Chem. 177*:751–766 (1949).
10. Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall, Protein Measurement with the Folin-Phenol Reagent, *J. Biol. Chem. 193*:265–275 (1951).
11. Standard Practice for Temperature Calibration of Differential Scanning Calorimeters and Differential Thermal Analyzers, *Annual Book of Standards*, ASTM, West Conshohocken, PA, 1984, ASTM E 967-83.
12. Standard Practice for Heat Flow Calibration of Differential Scanning Calorimeters, *Annual Book of Standards*, ASTM, West Conshohocken, PA, 1984, ASTM E 968-83.
13. Beveridge, T., S.J. Toma, and S. Nakai, Determination of SH- and SS-Groups in Some Food Proteins Using Ellman's Reagent, *J. Food Sci. 39*:49–51 (1974).
14. Hayakawa, S., and S. Nakai, Relationships of Hydrophobicity and Net Charge to the Solubility of Milk and Soy Proteins, *Ibid. 50*:486–491 (1985).
15. Wilkinson, L., Systat, in *The System for Statistics: SYSTAT*, Evanston, IL, 1990.
16. Wolf, W.J., Sulfhydryl Content of Glycinin: Effect of Reducing Agents, *J. Agric. Food Chem. 41*:168–176 (1993).
17. Hoshi, Y., and F. Yamauchi, Determination of Sulfhydryl and Disulfide Contents of Soybean 11S Globulin and Their Change by Lyophilization, *Agric. Biol. Chem. 47*:2435–2440 (1983).
18. Scilingo, A.A., and M.C. Añón, Calorimetric Study of Soybean Protein Isolates: Effect of Calcium and Thermal Treatments, *J. Agric. Food Chem. 44*:3751–3756 (1996).
19. Castellani, O.F., E.N. Martínez, and M.C. Añón, Structural Modifications of an Amaranth Globulin Induced by pH and NaCl, *Ibid. 46*:4846–4853 (1998).
20. Mata, L.L., Sánchez, D.R. Headon, and M. Calvo, Thermal Denaturation of Human Lactoferrin and Its Effect on the Ability to Bind Iron, *Ibid. 46*:3964–3970 (1998).
21. Ma, C.Y., and V.R. Harwalker, Studies of the Thermal Denaturation of Oat Globulin by Differential Scanning Calorimetry, *J. Food Sci. 53*: 531–534 (1988).
22. Kella, N.K.D., and J.E. Kinsella, A Method for the Controlled Cleavage of Disulfide Bonds in Proteins in Absence of Denaturants, *J. Biochem. Biophys. Methods 11*:251–263 (1985).
23. Hoshi, Y., and F. Yamauchi, Determination of Sulfhydryl and Disulfide Contents of Soybean 11S Globulin and Their Change by Lyophilization, *Agric. Biol. Chem. 47*:2435–2440 (1983).
24. Petruccelli, S., and M.C. Añón, Relationship Between the Method of Obtention and the Structural and Functional Properties of Soy Protein Isolates. 1. Structural and Hydration Properties, *J. Agric. Food Chem. 42*:2161–2169 (1994).
25. Scilingo, A.A., Propiedades estructurales y funcionales de aislados proteicos de soja modificados. Efecto de la presencia de calcio, Ph.D. Thesis, Universidad Nacional de La Plata, Argentina, 2000.

[Received May 23, 2002; accepted October 1, 2003]