Relationship between the Method of Obtention and the Structural and Functional Properties of Soy Protein Isolates. 1. Structural and Hydration Properties

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Soy protein isolates exhibit heterogeneous protein subunit compositions; their structural and functional properties are determined by the processing conditions. Drastic thermal conditions at pH 7 and 9 result in protein denaturation and polymerization, as evidenced by increased water retention capacity and lower solubility, surface hydrophobicity, and a higher level of AB−11S aggregates. Treatments of glycinin with urea and Na_2SO_3 at pH 7 incorporated 20% of sulfonate groups, resulted in no solubility losses of 11S protein A and B polypeptides, but increased their surface hydrophobicity. The increase of 7S fraction leads to an increase of aliphatic hydrophobicity. Thermal treatments at pH 7 and lower protein content lead to high solubility and high surface hydrophobicity isolates. 7S globulin was completely denatured, while 11S denaturation depended on the treatment conditions; different proportions of AB−11S, β−7S, and B−11S aggregates were formed. Thermal treatments at pH 9 favored dissociation and denaturation of AB−11S protein.

Keywords: *Hydration properties; soy proteins; protein functional properties; hydrophobicity; structural characteristics*

INTRODUCTION

Soy protein isolate contains 90% protein, its major components being 7S globulin or β-conglycinin and 11S globulin or glycinin. β-Conglycinin is a trimer formed from various combinations of the three subunits (α′, α, and β). Glycinin is made up of six subunits, each consisting of a basic polypeptide (B polypeptide) and an acidic polypeptide (A polypeptide) which are connected by a single disulfide bond forming the AB subunit (Badley et al., 1975). There is, however, a great heterogeneity among the minor components (Iwabuchi and Yamauchi, 1987a,b). Both glycinin and β-conglycinin are very complex oligomeric proteins, their association/dissociation state and thermal stability being highly dependent on the pH and ionic strength (Badley et al., 1975; Kitamura et al., 1976; Utsumi et al., 1981; Thanh and Shibasaki, 1979; Kakalis and Baianu, 1990; Iwabuchi et al., 1991a,b; Fukushima, 1991a,b). The structural similitude among the constituent domains of the subunits of these globulins leads to a tendency to association among some of the subunits (Yamauchi et al., 1991). The wide variety of protein species, as well as the structural complexity of the major components, makes it difficult to predict the structural and functional changes that would occur when the isolates are subjected to different treatments.

The possibility of using the values of certain structural properties, such as surface hydrophobicity (Kato and Nakai, 1980; Townsend and Nakai, 1983; Voutsinas et al., 1983a,b; Hayakawa and Nakai, 1985a,b; Nakai et al., 1991), extent of association/dissociation, stability, net charge, and molecular size (Kato, 1991), to predict protein functionality is being studied. Values of solubility and viscosity and numbers of free sulfhydryl groups and disulfide bond also give information about the protein structure. Solubility, for instance, reflects the balance of charges and hydrophobicity of the protein molecule (Bigelow, 1967; Hayakawa and Nakai, 1985), viscosity is related to steric effects and molecular interactions, and the number of SH_F and SS groups provides information about molecular flexibility and the ability to produce cross-linking (Nakai et al., 1991; Kato, 1991). Knowledge of the relationships that occur among these structural and functional properties is of interest to produce changes by means of the design of different treatments.

In our laboratory, we have studied the effect of thermal treatments under different sets of conditions—time, temperature, pH, and presence of reducing agents—on the degree of association/dissociation of soy protein isolates (Petruccelli and Añón, 1994a,b). The conditions under which aggregation of AB−11S, β-7S subunits, and B−11S polypeptides occurs have been determined. In this paper we describe how some of these treatments modify characteristic structural parameters such as surface and exposed hydrophobicity, numbers of SH_F and SH_T groups (SH_F + SS bond), degree of denaturation, and protein profile as well as their hydration properties (solubility, water retention). The aim of this study was to establish the relationships between treatment and structural modification and functional properties.

MATERIALS AND METHODS

Preparation of Isolates. Protein isolates were prepared from defatted soybean flour (Sanbra, S. A., Sao Pablo, Brazil) as previously described (Petruccelli and Añón, 1994a). The isoelectric precipitate thus obtained was suspended in water and neutralized with NaOH. After this initial procedure, the aqueous suspensions were subjected to different treatments, which will be described next.

Treatment 1 (Neutralization to pH 7 or 9). Protein concentration of the aqueous suspension was 10%. Proteinates obtained at either pH 7 or 9 were divided into three aliquots. No thermal treatment was applied to the first aliquot (isolates 1 and 4 for the proteinates at pH 7 and 9, respectively). The second and third aliquots were placed in a bath at 98 °C for either 5 (isolates 2 and 5) or 30 min (isolates 3 and 6), and they were cooled immediately in an ice bath. The temperature

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Table 1. Treatment Conditions of Protein Isolates

treatment	isolate	characteristic	thermal treatment	treatment, Na_2SO_3	dialysis
1	1	pH 7	–	–	–
	2	pH 7	98 °C, 5 min	–	–
	3	pH 7	98 °C, 30 min	–	–
	4	pH 9	–	–	–
	5	pH 9	09 °C, 5 min	–	–
	6	pH 9	98 °C, 30 min	–	–

treatment	isolate	composition	treatment, 6 M urea	treatment, Na_2SO_3	dialysis
2	7	normal	–	–	+
	8	normal	–	0.05%	+
	9	normal	+	0.05%	+
	10	rich in 7S	–	–	+
	11	rich in 7S	–	0.05%	+
	12	rich in 7S	+	0.05%	+
	13	rich in 11S	–	–	+
	14	rich in 11S	–	0.05%	+
	15	rich in 11S	+	0.05%	+

treatment	isolate	characteristic	thermal treatment	treatment, Na_2SO_3	dialysis
3	16	pH 7	80 °C	–	+
	17	pH 7	92 °C	–	+
	18	pH 7	–	2%	+
	19	pH 7	80 °C	2%	+
	20	pH 7	92 °C	2%	+

reached by isolates 2 and 5 was about 75 °C, while that reached by the isolates 3 and 6 was 98 °C. Samples were frozen and freeze-dried. These isolates were not dialyzed (Table 1).

Treatment 2. Three types of isolates were prepared, and the above-mentioned process was followed. Isolates 7–9 were neutralized to pH 7 (isolates 7–9). As far as the others are concerned, instead of carrying out the isoelectric precipitation at pH 4.5, precipitation was performed at pH 6.4 for 18 h at 4 °C. In this way, an isolate richer in 11S globulin was obtained (isolates 13–15). The supernatant of that precipitation was adjusted to pH 4.8, an isolate richer in the 7S fraction being thus obtained (isolates 10–12). These isolates were neutralized to pH 7 and divided into three aliquots, which then underwent different treatments. The first aliquot was left as control (samples 7, 10, and 13), the second was treated with 0.05% Na_2SO_3 (samples 8, 11, and 14), and the third one was treated with 0.05% Na_2SO_3 6 M urea (isolates 9, 12, and 15) for 3 h at room temperature in a shaker at 120 oscillations/min. The protein concentration at which this treatment was performed was 4%. The nine isolates obtained were dialyzed against separate lots of water (cutoff 12 400) for 48 h at pH 7 and were then freeze-dried (Table 1).

Treatment 3. The isolate was neutralized at pH 7 and divided into two parts. One was left as control (isolates 16 and 17), and 2% Na_2SO_3 was added to the other (samples 18–20). Protein concentration corresponding to this treatment was 4%. Each isolate was divided in turn into three aliquots. One of them was left untreated (isolate 18). Thermal treatment was applied to the other two: one of them was treated until a temperature of 80 °C was attained (isolates 16 and 19), whereas the other was brought to 92 °C (isolates 17 and 20). These treatments were carried out in a shaker at 120 oscillations/min. Once these temperatures were attained, the aliquots were immediately cooled in an ice bath. Times needed to reach these temperatures were 6 and 12 min, respectively. Isolates were dialyzed against distilled water for 48 h at 4 °C and were then freeze-dried (Table 1).

Differential Scanning Calorimetry. A DuPont 910 DSC was used for these studies. Hermetically sealed aluminum pans were prepared so as to contain 1.7–3.4 mg of isolate suspended in water (10–20%). These capsules were heated from 30 to 130 °C at a rate of 10 °C/min. An empty double pan was used as reference. Two duplicate runs were performed as a minimum.

Determination of Free Sulfhydryl Groups (SH_F). SH_F groups were determined according to the procedure of Beveridge et al. (1974) by dissolving 50 mg of isolate in 5 mL of a buffer containing 0.086 M Tris, 0.09 M Gly, 0.004 M EDTA, and 8 M urea, pH 8. 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 (about 15 min). Protein concentration was determined according to the biuret method (Gornall et al., 1949); bovine albumin dissolved in 8 M urea was used for plotting the calibration curve. Duplicate determinations were made.

Determination of Total Sulfhydryl Groups (SH_T). The determination of total sulfhydryl groups (SH_F + SS bonds) was performed according to the methods of Thannhauser et al. (1984) and Damodaran (1985) by mixing 70 μL of the isolate solution (10 mg/mL) and 1 mL of disodium 2-nitro-5-thiosulfobenzoate (NTSB), pH 9.5, prepared just before use. The color was left to develop for about 20 min in the dark, and the absorbance was determined at 412 nm, using test NTSB as reference. Duplicate determinations were performed. To obtain the disulfide bond concentration from the absorbance values, a coefficient of molar extinction of 13 600 M^{-1} cm^{-1} was used. Protein concentration was determined according to the biuret method (Gornall et al., 1949), using bovine albumin as standard.

The percentage of sulfonate group was calculated as

$$\%\ \text{sulfonate groups} = \frac{SH_T^C - SH_T^R}{SH_T^C} \times 100 \qquad (1)$$

where SH_T^C is the total sulfhydryl groups of the control isolate and SH_T^R is the total sulfhydryl groups of the reduced isolate.

Electrophoresis (SDS–PAGE). A continuous and dissociating buffer system was used, containing 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS for the separating gel and 0.025 M Tris-HCl, 0.192 M Gly, and 0.1% SDS, pH 8.3, for the buffer corresponding to the runs. A 5–15% polyacrylamide gradient was performed. The electrophoresis equipment used was a Bio-Rad mini-Protean; runs were performed at a constant voltage (200 V). Densitograms corresponding to each gel were obtained by means of a TLC Scanning CS-910 double-wavelength Shimadzu spectrodensitometer. Wavelengths used were 570 and 395 nm, respectively, for the sample and reference.

Electrophoresis was carried out on isolates directly dissolved in the sample buffer (1.0% SDS, 0.05% bromophenol blue, 40% v/v glycerol, 0.125 M Tris-HCl, pH 6.8) and on isolates dissolved in water or in an 8 M urea solution, both in the presence and in the absence of β-mercaptoethanol (5% v/v).

Determination of Surface Hydrophobicity (H_0). Isolates were dissolved in 0.1 M phosphate buffer, pH 7, at a concentration of 4 mg/mL for 30 min at 20 °C, with occasional stirring. The suspensions thus obtained were centrifuged at 10000*g* for 30 min at 15 °C. Serial dilutions were made with the same buffer at concentrations in the 0.0025–4 mg/mL range. Surface hydrophobicity was then determined by means of different probes, i.e., ANS, DPH, and CPA (Hayakawa and Nakai, 1985a,b; Kato and Nakai, 1980). Duplicate runs were done. Protein concentration was determined according to the method of Lowry et al. (1951); bovine albumin was used as standard.

Solubility in Water. Isolates were dissolved with occasional vortex agitation for 1 h at room temperature and at a 1% concentration. They were then centrifuged at 10000*g* for 30 min at 15 °C. Protein content in the supernatant was determined according to the biuret method. Duplicate determinations were made throughout.

Water Holding Capacity (WHC). WHC was determined by the difference between the weight of protein as obtained in the solubility determination and that of the precipitate remaining after centrifugation. By subtracting from this difference the amount of dissolved protein, the water volume (milliliters) remaining in the precipitate was obtained. By

Table 2. Degree and Temperatures of Denaturation

isolate	ΔH_{7S} (J/g)	ΔH_{11S} (J/g)	T_D 7S (°C)	T_D 11S (°C)
1	0.37 ± 0.02	0.59 ± 0.04	74.59 ± 0.24	90.69 ± 0.02
2	0	0.39 ± 0.07		90.96 ± 0.23
3	0	0		
4	0.21 ± 0.01	0.36 ± 0.04	74.82 ± 0.01	87.34 ± 0.02
5	0	0.17 ± 0.01		87.84 ± 0.01
6	0	0		
7	0.32 ± 0.06	0.57 ± 0.05	75.31 ± 0.36	89.74 ± 0.25
8	0.37 ± 0.01	0.62 ± 0.02	74.56 ± 0.24	90.68 ± 0.01
9	0	0		
10	0.43 ± 0.06	0.22 ± 0.01	74.31 ± 0.01	90.73 ± 0.01
11	0.44 ± 0.04	0.23 ± 0.05	74.67 ± 0.23	91.56 ± 0.24
12	0	0		
13	0.19 ± 0.04	0.87 ± 0.04	78.2 ± 0.20	91.93 ± 0.24
14	0.10 ± 0.01	0.51 ± 0.01	75.86 ± 0.48	92.67 ± 0.01
15	0	0		
16	0	0.19 ± 0.01		92.03 ± 0.48
17	0	0		
18	0.32 ± 0.04	0.54 ± 0.01	75.62 ± 0.24	89.62 ± 0.48
19	0	0.47 ± 0.02		88.42 ± 0.24
20	0	0.22 ± 0.02		89.14 ± 0.48

dividing this value by the initial mass of the isolate, the WHC was obtained defined as milliliters of H_2O per gram of isolate.

Viscosity. Solutions containing 10% of isolates in water were prepared. Viscosity was measured by means of a Haake RV2 viscosimeter, sensor system NV. A 0/2/1 program was used up to a value of 128 rpm. Duplicate determinations were carried out at 20 °C, and the apparent viscosity at 128 rpm was then calculated.

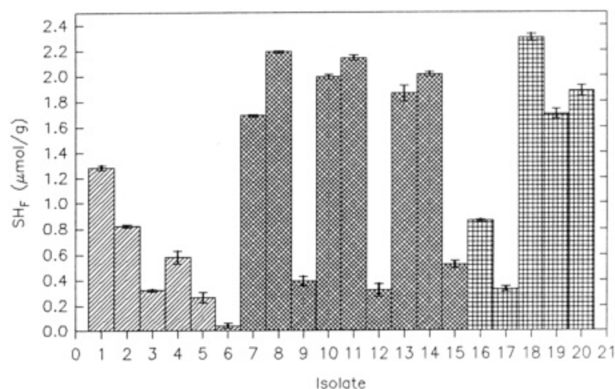
RESULTS AND DISCUSSION

The structural characteristics of the soy protein isolates were evaluated by measuring the extent of denaturation, number SH_F and SH_T groups, total and surface hydrophobicity, and changes on protein profiles. Table 1 shows the conditions under which the characterized isolates were obtained together with the number given to each isolate.

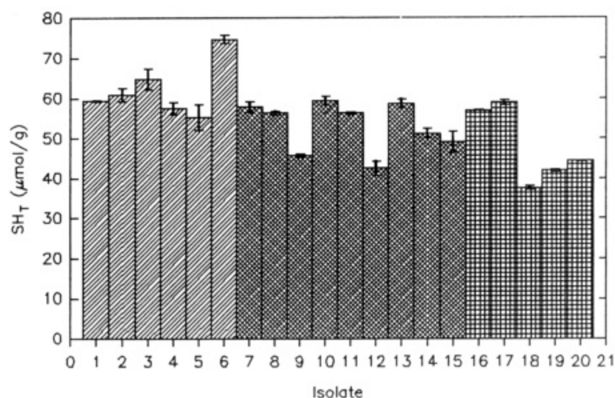
Denaturation Degree. The degree of denaturation of the isolates was determined by DSC. As can be seen, isolates subjected to thermal treatment at 98 °C for 30 min (isolates 3 and 6) or those which were heated to a temperature of 92 °C (isolate 17) were completely denatured, similarly to the isolates reduced in the presence of urea (isolates 9, 12, and 15) (Table 2). Isolates 2, 5, and 16 contain completely denatured globulin 7S, and 66.4%, 48.0%, and 32.8%, respectively, native 11S globulin. This would indicate that thermal treatments at pH 9 promote a higher degree of denaturation than do treatments at pH 7. A decrease of the denaturation temperature of glycinin when the pH is increased is observed as well (isolates 4 and 5).

When isolates 7 and 8 are compared (Table 2), no differences of either enthalpies or denaturation temperatures are noticed as a consequence of the Na_2SO_3, whereas those isolates that underwent a thermal treatment in the presence of 2% Na_2SO_3 ($\mu = 0.48$ M) show the stabilizing effect produced by the increase of ionic strength (Petruccelli and Añón, 1994a). As can be noticed, isolate 20 has a portion of 11S that shows no denaturation, whereas isolate 17, which underwent the same thermal treatment, exhibits total denaturation. Similar findings were obtained by comparing isolates 16 and 19.

In turn, isolates 18–20 exhibit a denaturation temperature of 11S globulin lower than that corresponding to the native isolate, resulting from the fact that the saline effect disappears because of the elimination of



1.1



1.2

Figure 1. Values of free sulfhydryl (SH_F) groups (1.1) and total sulfhydryl groups (SH_T) (1.2) of the isolates studied.

Na_2SO_3 by dialysis; thus, the partially reduced 11S fraction is more unstable than the native 11S.

Isolates enriched in 7S and 11S fractions by means of the precipitation of glycinin at pH 6.4 and of β-conglycinin at pH 4.8 exhibit the presence of both fractions. Isolate 10 contains 65.8% 7S and 34.2% 11S; isolate 13 has 17.0% 7S and 83.0% 11S. The usual isolates have a 7S and 11S content in the 36–63% range, respectively. These values were calculated by means of the enthalpies of denaturation, considering the total area corresponding to 7S and 11S.

Free Sulfhydryl and Total Sulfhydryl Content. Figure 1 shows the values of SH_F and SH_T. All isolates treated exclusively with Na_2SO_3 exhibit SH_F values higher than 2.0 μmol/g (isolates 8, 11, 13, and 18). Isolates that underwent only a thermal treatment exhibit a value of SH_F lower than 0.8 μmol/g. The normal value of an untreated isolate is 1.5–1.8 μmol/g.

Isolates 9, 12, and 15 have low values of SH_F groups, since they were completely denatured by urea; this leads to exposition of the inner SH_F groups, thus favoring their oxidation once the reducing agent has been removed. Isolates exhibiting the highest values of 7S and 11S (10 and 13) have SH_F values similar to that of a native isolate.

SH_T values should remain unchanged when a thermal treatment is applied, since oxidation of SH_F groups gives rise to disulfide bonds. Reduction with Na_2SO_3 leads to sulfonation and the RSO_3^- groups thus formed do not react with NTSB; then, the difference between the SH_T of the untreated isolate (control isolate) and the reduced isolate gives the degree of sulfonation attained during the reducing treatment. This difference is only significant when the number of sulfonate groups incorporated in the sample is important. Isolates 8, 11, and 14

exhibit a decrease of SH_T with regard to the controls (samples 7, 10, and 13) of 3.6%, 5.6%, and 13.7%, respectively. These values correspond to the percentage of incorporated sulfonate groups. Treatments performed in the presence of urea (samples 9, 12, and 15) increase sulfonation, the values being 19.2%, 21.2%, and 14.6%, respectively. As far as isolates 18, 19, and 20 are concerned, sulfonation, as compared with an isolate with no treatment (7), reached values of 37.1%, 28.3%, and 24.4%, respectively. In this case, the thermal treatment does not improve reduction, since it is not able to improve the access of sulfite due to the stabilizing effect that occurs as a result of the increased ionic strength (Petruccelli and Añón, 1994a).

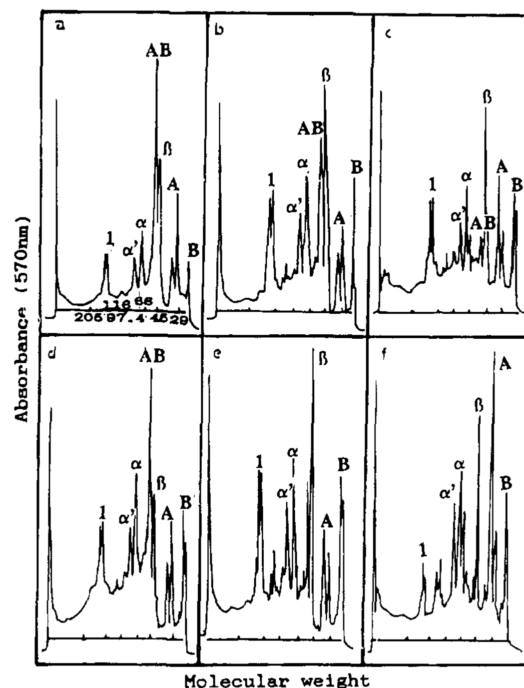
Treatments performed with 0.05% Na_2SO_3 yielded a very low sulfonation degree, because the low amount of sulfite does not make possible a good progress of the reduction reaction (Petruccelli and Añón, 1994a). A complete reduction of the AB dimers was achieved when the reducing treatment was performed in the presence of urea; instead, sulfonation remained low (only 8–15 μmol of RSO_3^-/g of isolate was incorporated). A better sulfonation is attained with 2% Na_2SO_3: 21, 16, and 14 μmol of RSO_3^-/g are incorporated. Only small modifications occur then in the net charge of the isolate as a result of sulfonate uptake. In the case of isolates 9, 12, and 15 sulfonation affects mainly the SS bonds involved in the AB binding, whereas in the case of isolates 18–20 the effect is mainly superficial since the globular structure is already stabilized.

Electrophoretic Profiles. Figure 2 shows the electrophoretic profiles of isolates 1–6. Fractions soluble in a 0.1% SDS buffer are shown in part 2.1, while those soluble in water appear in part 2.2. An aggregation of the AB subunit is noticed in those treatments carried out at 98 °C; this aggregation was partial in the treatment lasting 5 min, and total in treatments lasting 30 min. If the thermal treatments are carried out at pH 9, there is also a breakdown of AB, since the proportions of the peaks of A and B polypeptides are increased in relation to B (Petruccelli and Añón, 1994b). The fractions soluble in urea buffer of these isolates have the same composition as those soluble in SDS buffer.

With regard to the profiles of the water-soluble fractions, the most remarkable differences are observed in the time range 5–30 min of treatment at 98 °C performed at either pH 7 or 9. The former have the same composition and proportion of soluble subunits as those exhibited with SDS, whereas the latter have a high proportion of A polypeptide and a considerable decrease of B–11S polypeptide and β–7S subunit (Figure 2.2). Therefore, treatments lasting 30 min produce β–7S/B–11S aggregates, through interactions that break by the action of either urea or SDS; these aggregates are not soluble in water.

Figure 3 shows the electrophoresis of samples 7–15. An increase of the proportion of AB in those isolates having a higher content of 11S (isolates 13–15) in relation to an isolate of normal composition (sample 7) can be observed. A decrease in the proportion of that subunit in isolates having a higher 7S content (isolates 10–12) is also observed. Treatments with Na_2SO_3 lead to an increase of α′ and α subunits, while there is no increase of A and B polypeptides. Reduction in the presence of urea produces a complete dissociation of the AB subunit (isolates 9, 12, and 15). Electrophoresis of water-soluble fractions, performed either in the presence or in the absence of mercaptoethanol, shows that none of these isolates exhibit aggregates of B–11 or β–7S and that the A and B polypeptides produced by the action of Na_2SO_3 in the presence of water are soluble in water.

Figure 4 shows the densitograms of samples 16–20. No differences among the profiles of the fractions soluble in water, urea, and SDS are observed. Isolates with no reducing treatment (samples 16 and 17) exhibit aggregate I, which is not present in the reduced samples; moreover, isolate 16, which had been treated at 80 °C, has the AB subunit, whereas in isolate 17 (treatment



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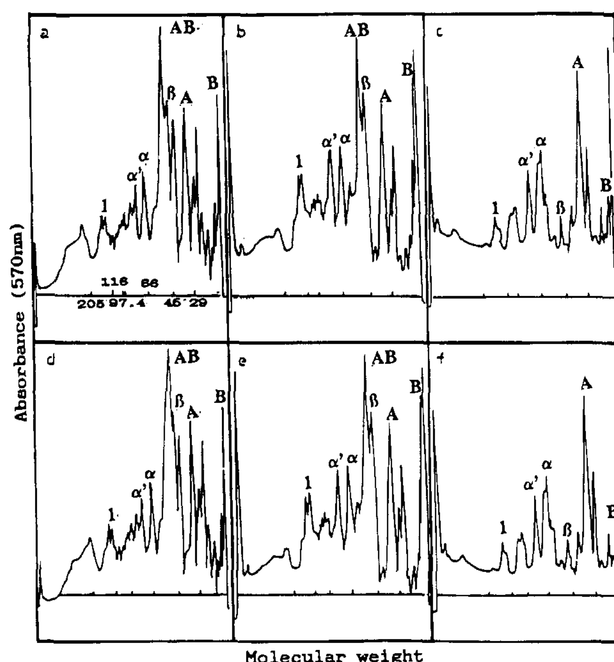


2.2

Figure 2. Densitometry scans corresponding to a SDS–PAGE electrophoresis in a 5–15% polyacrylamide linear gradient in the absence of β-mercaptoethanol: (2.1) fractions soluble in a buffer containing 0.1% SDS; (2.2) water-soluble fractions. Isolates: 1 (a); 2 (b); 3 (c); 4 (d); 5 (e); 6 (f).

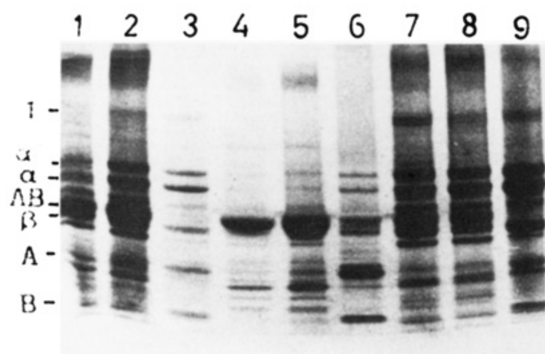


Figure 3. SDS–PAGE electrophoresis in 5–15% polyacrylamide gradient, in the absence of β-mercaptoethanol, corresponding to isolates 7, 8, 9, 13, 14, 15, 10, 11, and 12 for lanes 1–9 respectively.

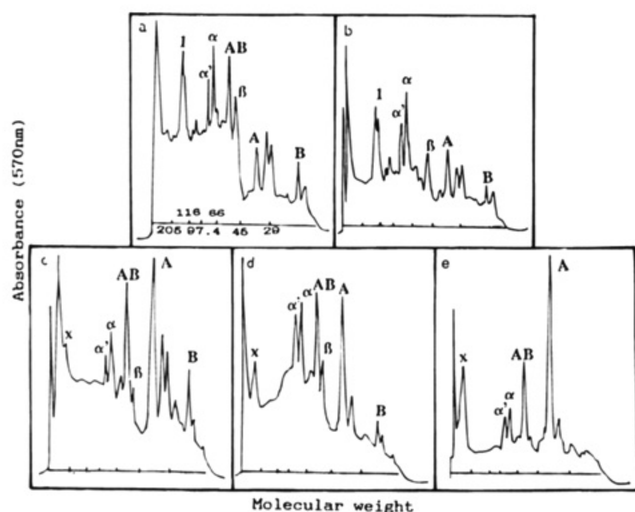


Figure 4. Densitometry scans corresponding to a SDS–PAGE electrophoresis, in 5–15% polyacrylamide linear gradient, in the absence of β-mercaptoethanol of the fractions soluble in buffer containing 0.1% SDS of isolates 16 (a), 17 (b), 18 (c), 19 (d), and 20 (e).

at 92 °C) this subunit is lacking, since it is fully aggregated. None of the isolates reduced with 2% Na_2SO_3 underwent aggregation of AB, since that concentration stabilizes the glycinin structure; thus, treatment at 92 °C produces only a partial denaturation of that structure. These isolates, in turn, show an increase of the proportion of A polypeptide—not accompanied by an increase of B polypeptide—and a decrease of β subunit. The presence of an aggregate of high molecular weight (X) is also noticed; this is not observed in samples without Na_2SO_3. The composition of the aggregate was determined by bidimensional electrophoresis, and results showed that it is formed by A and B polypeptides of glycinin (Figure 5). The presence of A and B aggregates of smaller molecular size than those formed by thermal treatment in the absence of sulfite could be imputed to surface sulfonation and/or lack of opening of the globular structure produced by this sulfite concentration, thus limiting possibilities of polymer formation.

All isolates subjected to thermal treatments have SDS-soluble aggregates that are sensitive to the action of mercaptoethanol and are constituted by subunits α and α′ of β-conglycinin and by A and B polypeptides of glycinin. These aggregates have a molecular weight higher than 300 000 since they are unable to penetrate into a 5–15% acrylamide gel.

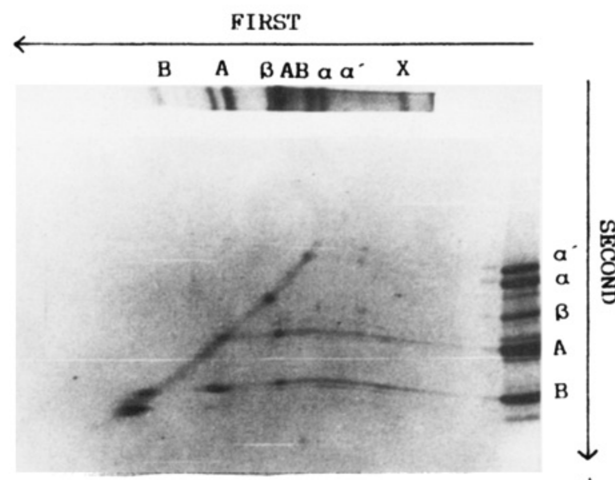


Figure 5. Bidimensional electrophoresis (first dimension SDS–PAGE without β-mercaptoethanol; second dimension SDS–PAGE with β-mercaptoethanol) of an isolate treated with 2% Na_2SO_3.

Surface Hydrophobicity. Hydrophobicity of a protein is an important structural property since it makes possible it to predict its behavior in interfaces. It can be assessed by means of probes that become fluorescent when they are in a nonpolar environment. Parameters used to quantify the probe–protein interaction are the magnitude of the wavelength shift of maximal emission toward the blue, the quantum yield, the affinity constant, and the number of binding sites (Cardamone and Puri, 1992). Another method is the use of the slope of the straight line obtained by plotting the intensity of relative fluorescence as a function of the protein concentration (Kato and Nakai, 1980). That slope is a function of the number of binding sites and of the nature and amount of the groups forming these sites, since they determine both the affinity and the energy transferred to the probe. The determination of the surface hydrophobicity (H_0) was performed with ANS, CPA, and DPH and that of the total hydrophobicity (H_T) with ANS. In this way, how the treatments applied affect the structure of the soy isolates obtained could be evaluated and, in turn, the changes observed could be related with the observed functional modifications. As a measurement of H_0, the initial slope of the line obtained by plotting intensity of relative fluorescence versus protein concentration is taken, since the increase of protein concentration favors the protein–protein interaction, thus avoiding the binding of the probe. In the case of ANS used as probe, the linear range is greater than with DPH and CPA; therefore, the aliphatic residues that interact with these probes (Hayakawa and Nakai, 1985b) would be the first to favor the protein–protein interaction. Figure 6.1 shows the H_0 of the isolates as determined with ANS. The highest H_0 values are those of isolates 16 and 17, which were exposed to thermal treatments at 80 and 92 °C, respectively; the former exhibited denaturation of 7S globulin, while in the latter both 7S and 11S globulins are denatured. Those thermal treatments were short, and the solubility loss was small. Instead, isolates 3 and 6, which underwent thermal treatment at a higher protein concentration, show a lower hydrophobicity than a native isolate (samples 1 and 7), probably resulting from aggregation and insolubilization of the protein as a consequence of hydrophobic regions which became exposed by the treatment. Isolates 9, 12, and 15 also exhibit a high H_0, since they had already undergone a treatment with urea and were then dena-

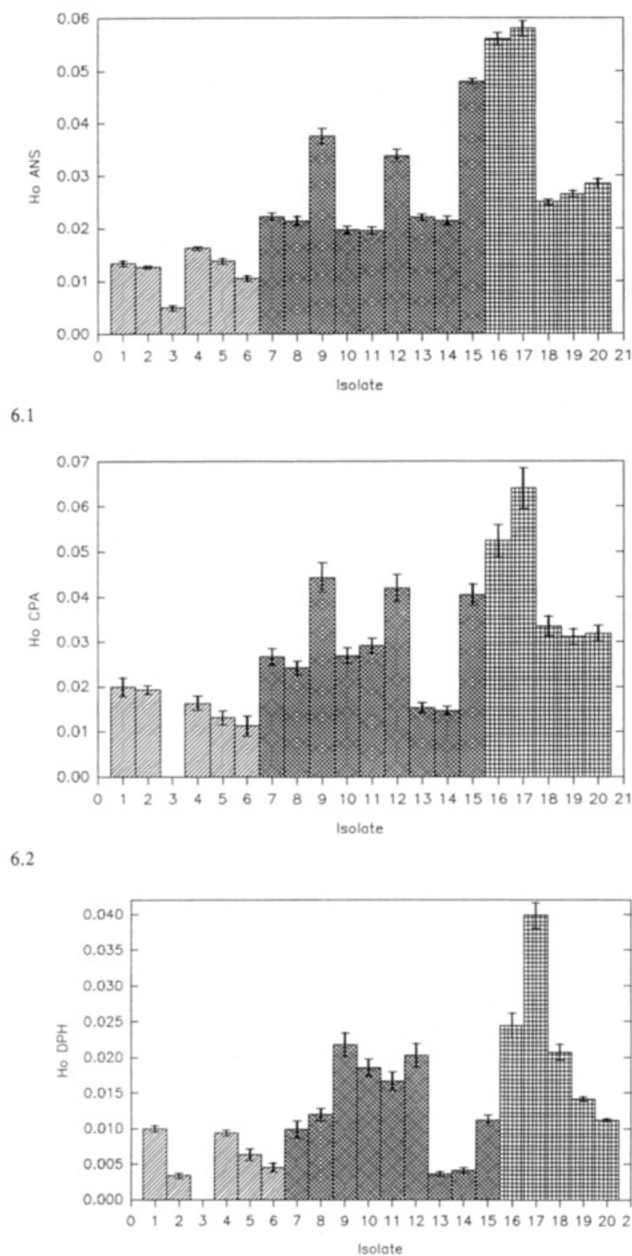


Figure 6. Values of surface hydrophobicity (H_0) determined with ANS (6.1), CPA (6.2), and DPH (6.3) of the isolates studied.

tured. H_0 values of isolates treated with Na_2SO_3 (8, 11, 14, 18–20) are similar to that of a native isolate. No significant differences were found between isolates having the highest content of 7S and 11S (samples 10 and 13, respectively) and the native form (isolate 10). Thermal treatments performed in the presence of 2% Na_2SO_3 (isolates 19 and 20) did not produce denaturation or increase hydrophobicity, as observed in the control sample (samples 16 and 17). Parts 2 and 3 of Figure 6 show the H_0 of isolates obtained with CPA and DPH, respectively. Samples exhibiting the highest hydrophobicity—with CPA and ANS as well—are isolates 9, 12, and 15–17. Results with DPH are similar, except for sample 15, which, similarly to samples 13 and 14, has a higher 11S content and a lower H_0. Instead, isolates having a higher 7S content (10 and 11) are more hydrophobic than a normal isolate (7 and 8). Isolates 19 and 20, which underwent a thermal treatment in the presence of Na_2SO_3, show a lower H_0 with DPH than those obtained with either ANS or CPA. Isolates with higher 7S content show an increase of aliphatic hydrophobicity in comparison with a native isolate, since the H_0 values are higher when either CPA or DPH is used. Instead, isolates richer in 11S show a lower H_0 with DPH, but the values of H_0 with ANS and CPA are similar to that of the native isolate; then, they could have a decreased aliphatic hydrophobicity. In these isolates the interaction with CPA would not be affected by this decrease of exposed aliphatic groups, perhaps as a consequence of the effect of the probe charge on the protein–probe interaction.

Figure 7 shows the relationship among the H_0 values of the isolates as determined with ANS, CPA, and DPH. The best correlation is observed between the values of H_0 determined with ANS and CPA ($r = 0.852$) with respect to those determined with DPH and CPA ($r = 0.820$) or DPH and ANS ($r = 0.606$).

From the above-described considerations it can be stated that the highest H_0 values—determined by ANS, CPA, or DPH—correspond to isolates denatured by either heat or urea with no solubility loss. Furthermore, the fact of presenting these hydrophobicity values points to a certain degree of recovery of the structure, since the probe required a hydrophobic environment to become fluorescent (Damodaran, 1986, 1987; Damodaran and Song, 1988), in spite of the fact that some of these isolates were completely denatured. Measurements of the dissociation constants of the probe–protein complexes of some of the isolates (1, 7, 9, 16, and 17) were performed, and no significant differences were found among them. Dissociation constants are about 30 μM, and it can be thought that the modifications induced by the treatments affect mainly the number of binding sites. However, the use this type of parameter to characterize the isolate is difficult because of the complexity of the system.

H_T was also evaluated in isolates completely denatured by thermal treatment in the presence of SDS to avoid aggregation (results not shown). The determination was carried out with ANS in the presence of SDS. A decrease of the probe affinity for the protein was observed in the presence of SDS ($K_d = 80$ μM). No changes of H_T were observed as a result of the treatments used or from modifications of the 7S and 11S content, except for the isolates 3 and 6, which showed a decrease of H_T, since most of the protein components are insoluble.

Solubility and Water Holding Capacity. The hydration characteristics of the isolates studied were also analyzed. Figure 8 shows the values of solubility in water and of the WHC of the isolates. It can be noticed that thermal treatments at 98 °C lead to a very important loss of the solubility. This decrease is in agreement with the degree of aggregation observed by electrophoresis and the hydrophobicity drop, since these regions would promote aggregation.

In isolates treated at both 80 and 92 °C, the solubility remains practically unaffected, since the thermal treatments were very short. In spite of this fact, the isolates have a higher H_0 and therefore a higher tendency toward aggregation. Intense aggregation, through disulfide bonds, such as that exhibited by isolates treated at 98 °C, requires a greater energy input than that provided in this case.

Thermal treatments at pH 9 produce dissociation besides aggregation, which makes it possible to explain

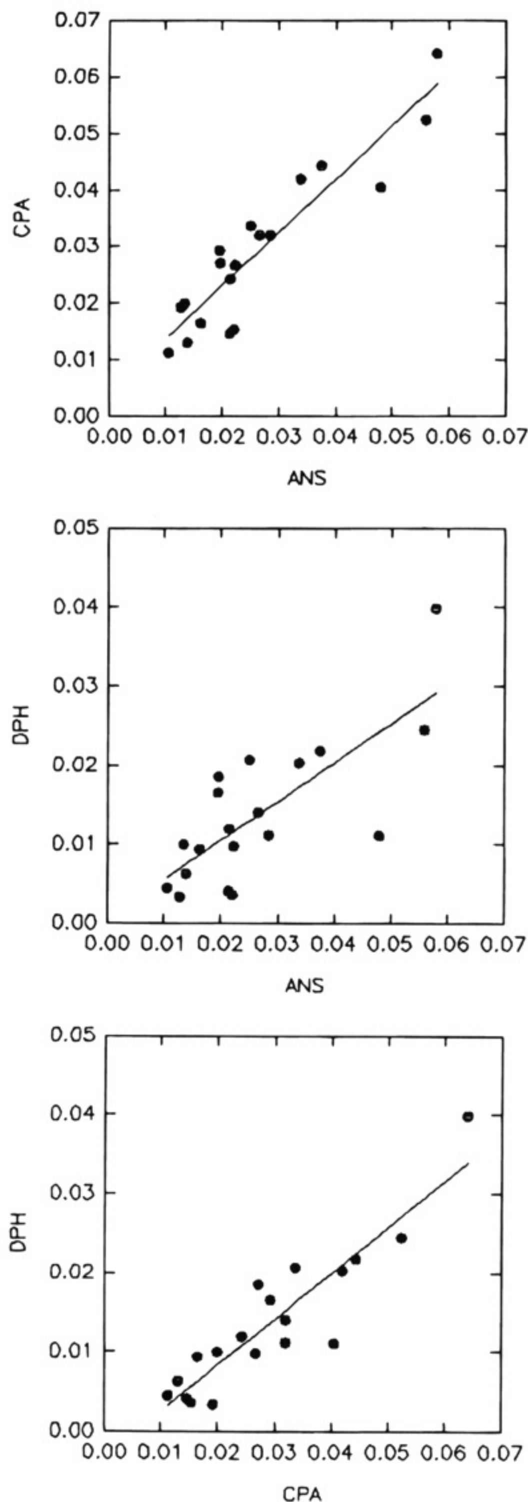
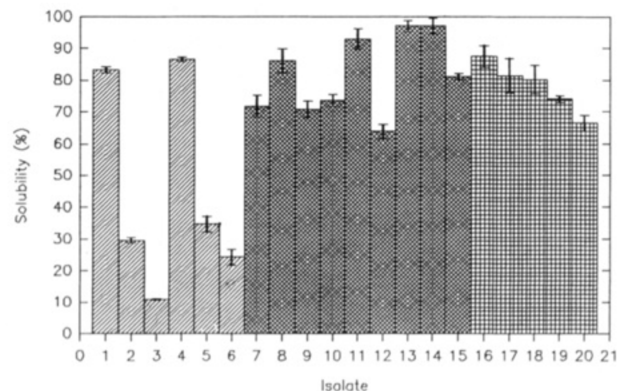


Figure 7. Comparison of surface hydrophobicity values obtained by three methods.

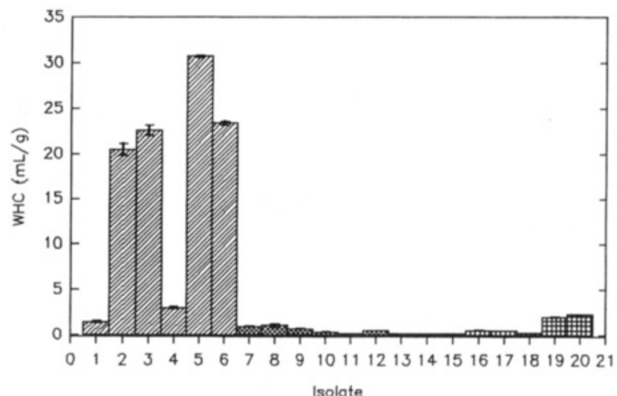
the higher solubility in comparison with those undergoing thermal treatment at pH 7.

The reducing treatment with Na_2SO_3 on the solubility has a variable effect, since an increase can be noticed in some isolates (8, 11, and 18), whereas a decrease is observed in others (isolates 19 and 20). However, these modifications are small.

Treatments with Na_2SO_3 and urea (isolates 9, 12, and 15) produce a solubility loss with respect to both untreated isolates and those treated with sulfite alone. Moreover, it is also observed that the isolate containing



8.1



8.2

Figure 8. Percent solubility in water (8.1) and water retention capacity (8.2) of the isolates studied.

a higher proportion of 11S is more soluble in water than an isolate having a normal composition.

Isolates 2, 3, 5, and 6 have the highest WHC values (Figure 8.2), being 20.5, 22.6, 30.7, and 23.4 mL of H_2O/g of isolate. These isolates were subjected to thermal treatment at high protein concentration, having thus a higher degree of aggregation and a lower solubility. When the thermal treatment is performed at pH 9, the increase of the WHC is larger than that observed in the treatment at pH 7. The increase of the duration of the thermal treatment from 5 to 30 min produces, in the case of isolates at pH 9, a decrease of the WHC, which is in accordance with the greater degree of dissociation observed in the electrophoresis runs.

Viscosity. Studies on the viscosity of isolates showed a high viscosity only in the case of isolates 2, 3, 5, and 6, their values being 125, 94, 146, and 179 mPa·s, respectively. This high viscosity correlates with their capacity to hold water (Figure 9).

Structural and Functional Properties. Figure 10 shows the relationship between viscosity and solubility in water as well as between viscosity and H_0, as determined with ANS. A decrease of viscosity is observed as the solubility increases. All isolates—with the exception of isolate 3—exhibit the same trend; isolate 3 has a very low solubility and a high degree of aggregation, even higher than that of isolate 6, since the latter shows both aggregation and dissociation, thus becoming more soluble. Isolates with a high H_0 tend to have low viscosity. It is possible to notice two different types of behavior: those isolates that went through the same thermal treatment are located on the same curve, whereas those which were not heated appear on a different curve (Figure 10). The most important modi-

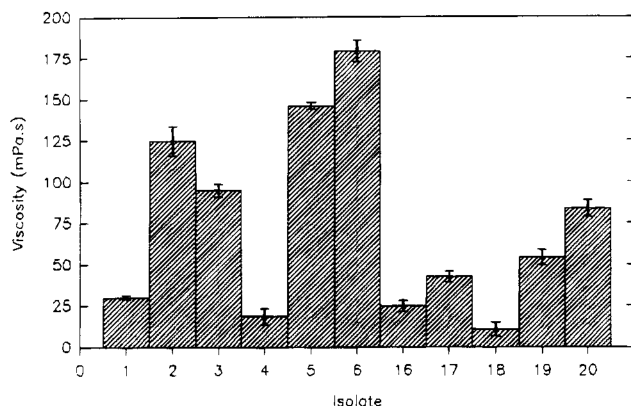
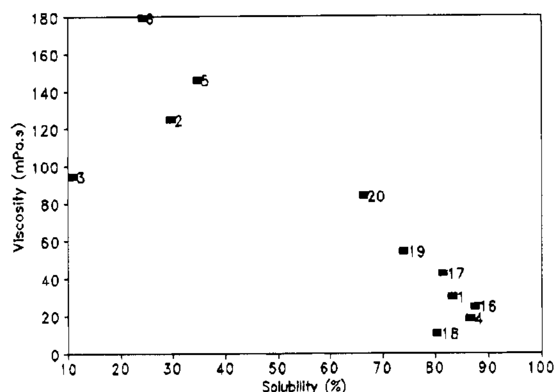
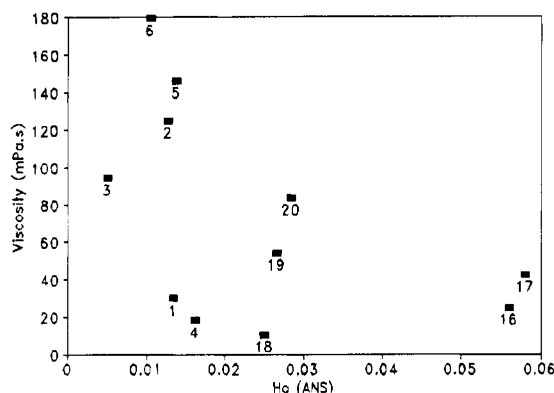


Figure 9. Viscosity (mPa·s) of the isolates studied.



10.1



10.2

Figure 10. Relationship between viscosity (mPa·s) and solubility in water (10.1) and between viscosity and surface hydrophobicity determined with ANS (10.2).

fications of viscosity are produced by the presence of insoluble aggregates. The soluble fractions have similar hydrodynamic volumes, in spite of having a different H_0 values, which could favor both the formation of soluble aggregates and the decrease of the WHC of the protein.

CONCLUSIONS

Long thermal treatments at pH 7 and 9 produced, at high protein concentrations, both aggregation and loss of solubility, leading to an increase of both the WHC and the viscosity of the isolate. These isolates had completely denatured 7S and 11S globulins, aggregates of the AB−11S subunit linked by disulfide bonds, and β−7S and B−11S having both disulfide bonds and bonds susceptible to the action of urea and SDS. They also had a low H_0 values.

Short-term thermal treatments at pH 7 and 4% protein concentration resulted in high solubility and H_0 isolates. Depending on whether the isolate reached the denaturation temperature of the 11S fraction or not, either completely denatured 7S and 11S globulins or partially denatured 11S globulins were obtained. These isolates exhibit the AB−11S subunit aggregated through disulfide interactions, partially in the former instance and totally in the latter. Aggregates β−7S/B−11S are also found.

Thermal treatments led to a decrease of the SH_F content (<0.8 μmol of SH/g of protein). Normal values corresponding to an isolate with no thermal treatment were 1.5−1.8 μmol of SH/g of protein). If the isolates underwent a reducing treatment, the values of SH_F were higher than 2.0 μmol of SH/g of protein, except if they have experienced a simultaneous thermal treatment. Mild reducing treatments (0.05% Na_2SO_3) had practically no effect on the charge or on H_0.

Values of H_0 determined by either ANS or CPA were the same for isolates obtained with the different treatments, with the exception of those isolates richer in 7S, which exhibited a higher aliphatic hydrophobicity. Modifications produced by the treatments usually applied had a negligible effect on the H_T values, except in the presence of a high proportion of insoluble proteins. Modification of the content of either 7S or 11S had no effect on the H_T.

It was possible to obtain isolates with different H_0 values and solubility by selecting the conditions of the thermal treatment: temperature, time, pH, and protein concentration. Regarding the last-mentioned condition, a 10% concentration produced insolubilization and aggregation. Treatments at a 4% protein concentration produced an increase of the H_0 with no solubility loss. Treatments at pH 9 favored dissociation of the AB subunit during the thermal treatment, accompanied by greater denaturation. Reduction of glycinin in the presence of urea and 0.05% Na_2SO_3 led to a 15−20% sulfonation, with no loss of solubility of A and B polypeptides. These isolates present a high H_0. The increase of 7S content led to an increase of the aliphatic hydrophobicity.

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