Thermal Aggregation of Soy Protein Isolates

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Thermal behavior of soy protein isolates under different conditions of temperature, time, pH, protein concentration, and presence of reducing agents was studied. Thermal treatments above 85 °C showed a decrease in concentration of the AB-11S subunit and of the two protein species of 20 and 29 kDa, and a gradual increase in the concentration of the A and B polypeptides of glycinin. None of the thermal treatments tested led to modifications of the relative proportions either of the high molecular weight aggregates (100–200 kDa) observed in the electrophoretic profiles or of the α′ and α subunits of β-conglycinin. Increasing the pH to 9 or 10 and increasing the protein isolate concentration enhanced AB-11S aggregation during the thermal treatment. Either the presence of Na_2SO_3 or the pH 9–10 favored the β-β-conglycinin/B-glycinin aggregation. This interaction requires an increase of SH groups. Initially the β-β-conglycinin/B-glycinin aggregates were stabilized by hydrophobic interactions and later by SS bonds.

Keywords: *Soy protein isolates; thermal; aggregation*

INTRODUCTION

Heat is the most common physical agent with the ability to denature proteins. Heat denaturation leads to dissociation of proteins into their constituent subunits, unfolding of their structure, and surface exposure of their hydrophobic groups. Denaturation is usually accompanied by a decrease of solubility, which results from aggregation of the unfolded molecules, and by changes in certain functional properties, such as ability for gelification, foaming, and emulsification (Kinsella, 1982; Privalov, 1979; Wolf, 1970).

The association–dissociation phenomenon of protein induced by heat has been widely studied in fractionated soy proteins. In both glycinin (Wolf and Tamura, 1969; Yamagishi *et al.*, 1980, 1981, 1982; Mori *et al.*, 1982; Utsumi and Kinsella, 1985; Yamagishi *et al.*, 1987) and β-conglycinin (Mori *et al.*, 1986; Iwabuchi *et al.*, 1991a,b), denaturation depends on the prevailing conditions of pH, ionic strength, presence or sulfhydryl or disulfide groups, heating time, and temperature and rate of cooling. When glycinin is heated at 100 °C, ~50% of the protein is rapidly converted into a buffer-soluble aggregate. With continued heating, the soluble aggregates increased in size and precipitated (Wolf and Tamura, 1969; Mori *et al.*, 1982). The precipitated fraction consisted of the basic polypeptides, whereas the acidic polypeptides remained soluble (Mori *et al.*, 1982; German *et al.*, 1982). The precipitation reaction is accelerated when reducing agent is added. Iwabuchi *et al.* (1991b) found that with heating, β-conglycinin dissociated into its subunits that remain in their dissociated form unless salt is added to the system. This behavior has also been described for glycinin when thermal treatment is carried out in the presence of β-conglycinin, which inhibits the formation of glycinin B polypeptide aggregates as a consequence of β-conglycinin/B-glycinin preferential association (German *et al.*, 1982; Damodaran and Kinsella, 1982; Yamagishi *et al.*, 1983; Utsumi *et al.*, 1984).

Thermal behavior of these soy protein isolates has not been as thoroughly studied. The aim of the present study was to gain a better knowledge of the association stabilized through covalent bonds and the dissociation reactions of the major soy protein fractions resulting from thermal treatment under different conditions of temperature, time, pH, protein concentration, and the presence of reducing agents.

MATERIALS AND METHODS

Preparation of Isolates. Protein isolates were obtained from a defatted flour (Sanbra S.A., Brazil), which was dissolved in water at pH 8, made alkaline with 2 N NaOH (flour:water, 1:10, w/v) for 2 h at room temperature; pH was adjusted periodically. The suspension thus obtained was filtered through gauze, and the filtered material was centrifuged at 10000*g* for 30 min at 4 °C. The pH of the supernatant was adjusted to 4.5 with 2 N HCl. The precipitate formed was obtained by centrifugation at 5000*g* for 15 min at 4 °C. The precipitate was then suspended in water, and the pH was adjusted to 7. Protein concentration was determined by the Biuret method (Gornall *et al.*, 1949). Bovine albumin was used as standard.

Thermal Treatments. The soy protein isolates were subjected to treatments of different conditions of temperature, protein concentration, pH, and presence of reducing agents according to the following process. The protein isolate suspension was fractionated in 1-mL aliquots, which were placed in 1.5-mL Eppendorf tubes. Thermal treatments were carried out in a constant temperature bath. The heating rate was recorded with a thin Cu–constantan thermocouple, which was immersed in the protein suspension, and the thermocouple was connected to a recorder. An ice-water bath was used as reference. The Eppendorf tubes were removed from the thermostatic bath at different times and immediately cooled in an ice bath. After 1 h, an aliquot of each sample was taken to a concentration of 10 mg/mL with 0.1 M phosphate buffer (pH 7) containing 1% SDS. All the collected samples were extracted at room temperature for 1 h, with periodic vortex agitation. The samples were then diluted with the suitable sample electrophoresis buffer.

Effect of Temperature. Different aliquots of protein isolate, at a concentration of 70 mg/mL, were heated at 50, 60, 70, 80, 90, and 100 °C. The thermal history of each sample was recorded. For the first four thermal treatments, samples were removed at 1, 5, and 30 min, whereas samples corresponding to the two last temperatures were drawn either at short (1–15-min) or at long (20–90-min) heating times.

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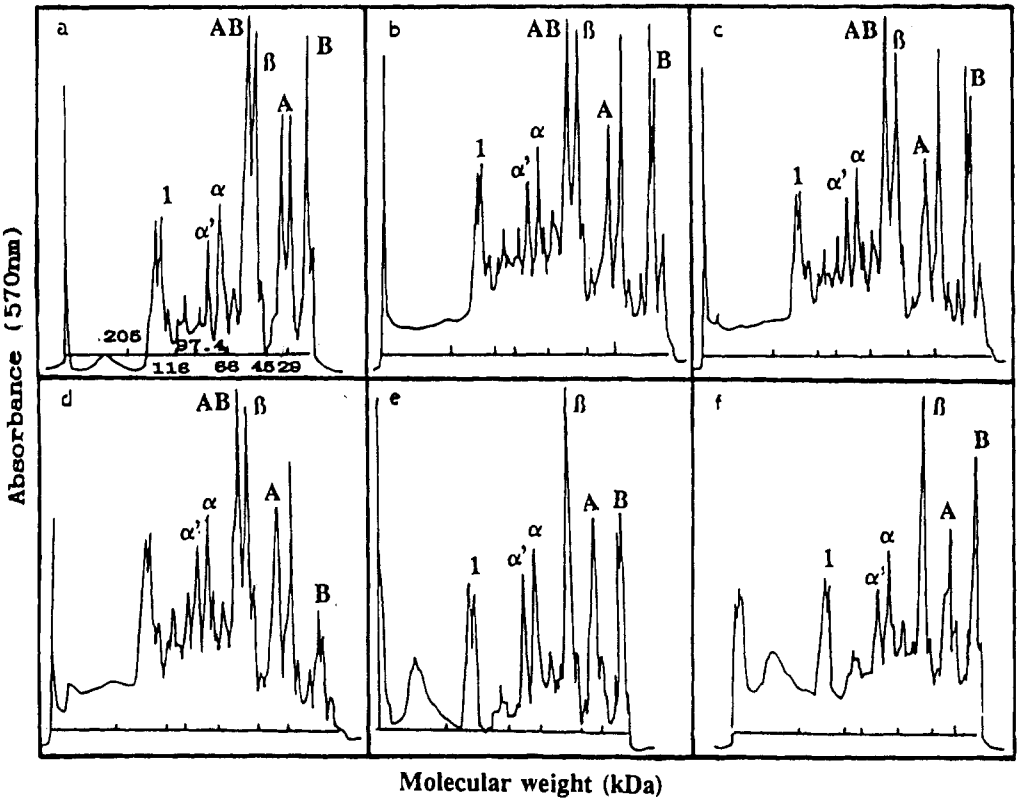

Figure 1. SDS-PAGE densitographies in a 5–15% linear gradient of soy protein isolates (70 mg/mL), (a) with no thermal treatment, or treated at (b) 60, (c) 70, (d) 80, (e) 90, and (f) 100 °C for 30 min.

Effect of Protein Concentration. Protein isolate dilutions in water were prepared with protein concentrations of 80, 60, 40, 20, and 10% of the initial concentration (70 mg/mL). The thermal treatment was then carried out at 100 °C for 6 min.

Effect of pH. The pH of the protein isolate was adjusted to pH 6 with 2 N HCl and to pH 8, 9, and 10 with 2 N NaOH. Thermal treatments were carried out at 90 and 100 °C for 1–15 min, at a protein concentration of 14 mg/mL.

Effect of the Presence of Reducing Agents. Samples of protein isolate (14 mg/mL) underwent a thermal treatment of either 90 or 100 °C at pH 7 in the presence of 0.01% Na_2SO_3. Samples were removed at 0, 5, 10, 15, and 30 min of treatment at 90 °C and at 0, 1, 2, 3, 5, 10, and 15 min of treatment at 100 °C. Each sample was analyzed by electrophoresis on polyacrylamide gels. The content of free and total sulfhydryl groups was also determined in some of the tests.

Determination of Sulfhydryl Groups. Total sulfhydryl (SH) groups (SH_T, SS bonds plus free SH groups) were determined according to the method of Thannhauser *et al.* (1984), whereas the free SH groups (SH_F) were evaluated according to the method of Beveridge *et al.* (1974). The Biuret method was used to determine protein concentration (Gornall *et al.*, 1949), with bovine albumin as standard. Determinations were performed in duplicate.

Electrophoresis. Electrophoresis was performed with a continuous and dissociating buffer system: 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS for the separating gel, and 0.025 M Tris-HCl (pH 8.3), 0.192 M glycine, and 0.1% SDS for the run buffer (Laemmli, 1970). A 5–15% polyacrylamide gradient was used. Densitographies corresponding to each gel were obtained with a TLC-scanning CS-910 double-wavelength Shimadzu spectrodensitograph. Wavelengths used were 570 and 395 nm for the sample and reference, respectively. Analyses were done in duplicate.

The electrophoretic profile was analyzed with the relative peak heights. This measure was independent of the amount of protein electrophoresed. Peak heights are expressed in relation to the β subunit of protein 7S because, according to our studies, this protein is less affected than the other soy proteins by the thermal treatment. Percentages of aggregation were determined by the relationship between the AB-11S/β-7S relative peak height of a thermal-treated isolate and that

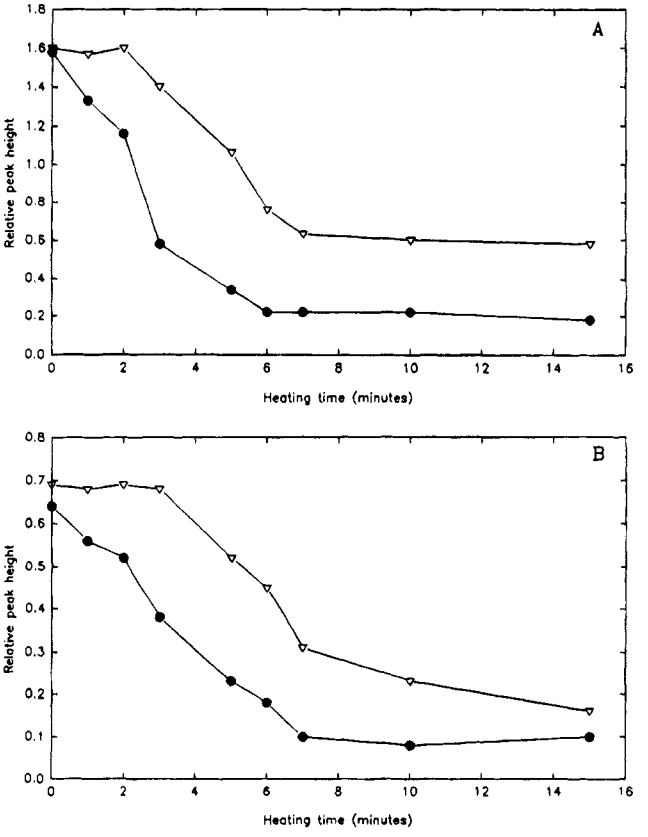

Figure 2. Variations of the peak height of (A) the AB-11S intermediate subunit and of (B) the protein species 29 kDa/β-7S ratio as a function of the heating time at (▽) 90 and (●) 100 °C.

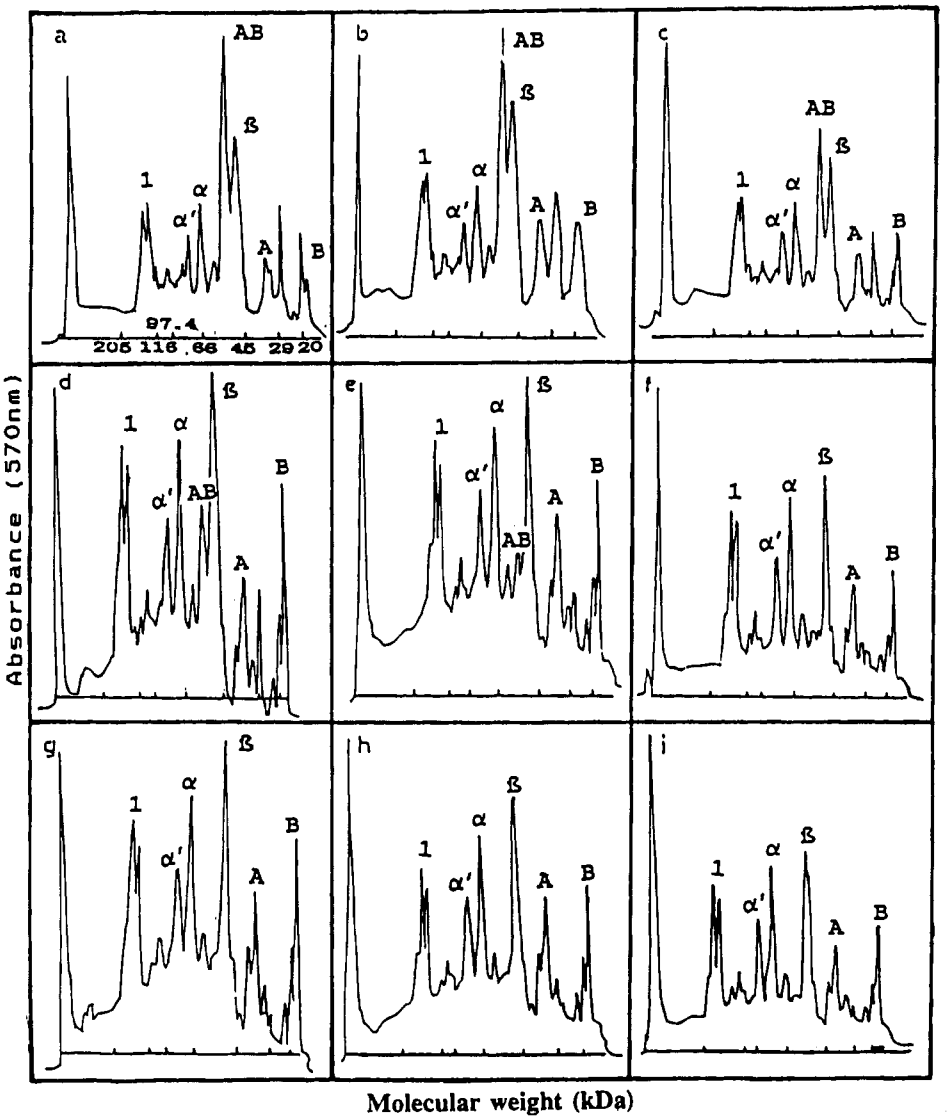

Figure 3. SDS-PAGE densitographies in a 5–15% linear gradient of soy protein isolates (70 mg/mL) treated at 100 °C for different periods of time: (a) 0 min, (b) 1 min, (c) 2 min, (d) 3 min, (e) 5 min, (f) 6 min, (g) 7 min, 10 (h) 10 min, and (i) 15 min.

of an unheated isolate:

$$\frac{(\text{AB-11S}/\beta\text{-7S})_{\text{heated isolate}}}{(\text{AB-11S}/\beta\text{-7S})_{\text{unheated isolate}}} 100 \qquad (1)$$

RESULTS AND DISCUSSION

Samples of soy protein isolates (70 mg/mL) underwent thermal treatments at different temperatures (50–100 °C) for up to 30 min. Analysis of the protein profiles as obtained by electrophoresis in the absence of 2-mercaptoethanol (Figure 1) show no significant differences when the thermal treatment is carried out at temperatures below 80 °C. Under these conditions of temperature (<80 °C), although the proteins of the isolate have been denatured [partial denaturation of the 11S and total denaturation of the 7S fraction, as determinated by differential calorimetric scanning (DSC)], no aggregation was observed. Similarly, Yamagishi *et al.* (1980) did not observe aggregation of the AB-11S subunits when a 0.5% glycinin suspension (0.1 M ionic strength) was treated at 70 °C Modifications are detected at 90 and 100 °C in the bands corresponding to the AB-11S subunit, to the A and B polypeptides of glycinin, and to the two protein species with molecular weights of of 20 and 29 kDa. The 20 kDa protein species could correspond to the fraction termed 7S basic globulin (Sathe *et al.*, 1987) or to the trypsin inhibitor. As far as the 29 kDa polypeptide concern, it could be a part of the A-11S species. Variations of the peak height of the AB-11S intermediate subunit and of that corresponding to the 29 kDa protein species, each with reference to subunit β-7S, are shown in Figure 2. In these species, the first modifications at 100 °C are detected at 2 min. The modifications become remarkable between 3 and 6 min, and become milder after 7 min of heating. Densitographs corresponding to the different thermal treatments performed at 100 °C also show, for heating times of >2 min, a decrease of the 20 kDa protein species and a gradual increase of the A and B polypeptides of glycinin (Figure 3). At 90 °C the modifications just mentioned take place at longer heating times and to a lesser extent. Residual values of the AB-11S subunit, in relation to the AB-11S/β-7S height of an unheated isolate, as described under Materials and Methods, are ~39 and 13%, after a 15-min treatment at 90 and 100 °C, respectively. At longer heating times (30 min), aggregation is ~87%, regardless of the heating temperature (results not shown). An important increase of the B polypeptide of 11S is observed as well; its peak height becomes greater than that corresponding to the β-7S

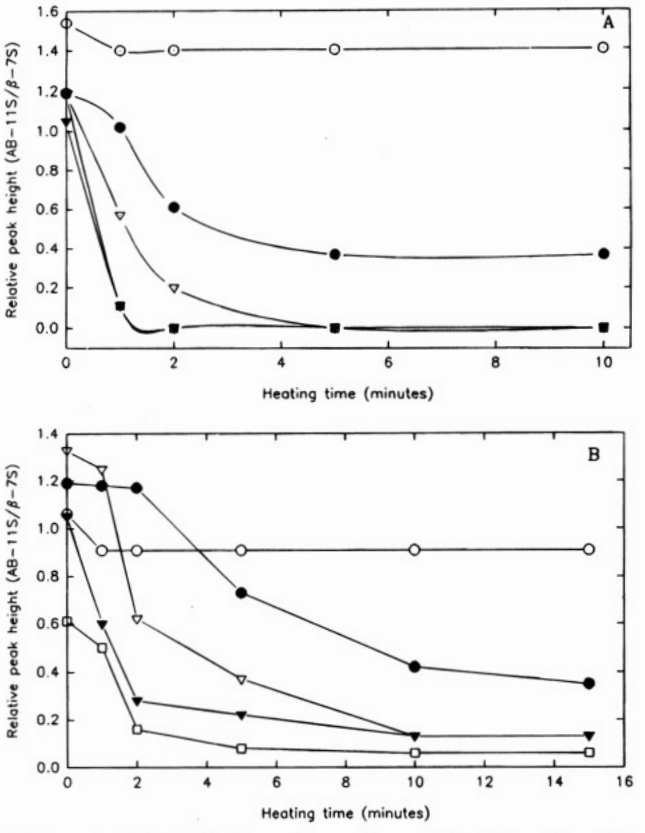

Figure 4. Effect of pH on the ratio peak height of the AB-11S intermediate subunit/β-7S as a function of heating time at (A) 100 °C and (B) 90 °C and pH 6 (○), pH 7 (●), pH 8 (▽), pH 9 (▼), and pH 10 (□). The concentration of protein used was 14 mg/mL.

subunit. This increase is accompanied by an increase of the A-11S polypeptide, even though the latter is 50% lower than that of the B-11S polypeptide. This result is in accordance with that obtained by Yamagishi et al. (1983), who noticed that treatment of glycinin under non gelation conditions is accompanied by dissociation of the AB-11S subunit. However, our results show that when in a short period of time the temperature reached by the sample overpasses the one for glycinin denaturation, aggregation of intermediate AB subunit without any dissociation is observed.

None of the thermal treatments tested led to modifications of the relative proportions of either the high molecular weight aggregates (100−200 kDa) observed in the electrophoretic profiles (Figures 1 and 3), or of the α′, α, and β subunits of β-conglycinin. Presence of aggregates, formed by α and α′-7S subunits, at temperatures higher than that of denaturation of β-conglycinin suggest that these aggregates are stable to the thermal treatments applied or they form again during cooling. These results agree with those obtained by Utsumi *et al.* (1984), but are not in accord with those reported by Iwabuchi *et al.* (1991b) who consider that α′ and α-7S subunits are the most polar and the most unstable when interacting with each other. Moreover, the possiblity that Iwabuchi *et al.* (1991b) might have lost the αα′ aggregates during the subunits purification process deserves some consideration.

Study of the recorded heating rate show that the first changes in the aggregation−dissociation state of the soy proteins present in the isolates occur when the temperature is >85 °C. Kinetic parameters corresponding to heat denaturation of soy protein isolates obtained in our laboratory by DCS show that treatment at 85 °C for 5

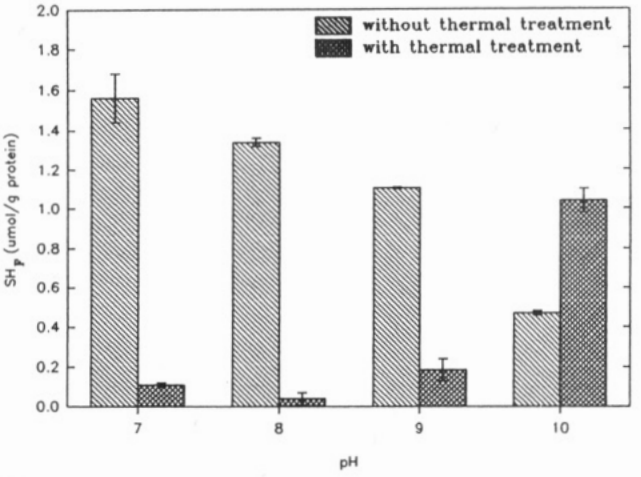

Figure 5. Variation of content of free SH groups as a function of pH at different heating times at 100 °C during a 90-min period.

min leads to 69% denaturation of glycinin (Arrese, 1991). This result indicates the need for a complete denaturation of glycinin and β-conglycinin to obtain aggregation reactions. Aggregation of the AB-11S subunit is enhanced by increasing the protein concentration at which the thermal treatment is performed. Percentages of aggregation obtained, as described under Materials and Methods, after heating for 6 min at 100 °C were ~64, 76, and 85% for protein concentrations of 7, 28, and 70 mg/mL, respectively.

The effect of pH on the aggregation−dissociation reactions is shown in Figure 4. In this case, thermal treatments were carried out at 90 and 100 °C for 1−15 min. Protein concentration was 14 mg/mL. At 100 °C, aggregation of the AB-11S subunit is favored as pH is increased from 7 to 10. Under these conditions, short heating times are needed (5 and 2 min at pH 8 and pH 9 and 10, respectively) for disappearance of the AB-11S peak in the electrophoretic profile. No aggregation could be detected at pH 6; at this pH, ionization of SH groups is negligible and, thus, the SH/SS interchange is reduced.

Longer heating times are required at 90 °C, but total disappearance of the AB-11S peak was not achieved at any of the pH values studied. Changes observed in the 20 and 29 kDa protein species are also favored by a pH increase. In addition, rupture of the AB-11S subunit was observed after a 2-min treatment at 100 °C at pH 9 or 10. After 2 min of this treatment, a decrease of the bands corresponding to both the β-7S subunit and the B-11S polypeptide appeared, suggesting the formation of β-7S/B-11S aggregates. This aggregation occurs at pH 8, but to a lesser extent, and was no longer observed at pH 7 (results are not shown). These results indicate that in the absence of sulfhydryl compounds, β-7S/B-11S aggregation is favored under pH values where both the β-7S subunit and B-11S polypeptides are negatively charged. This conclusion does not agree with the results of Damodaran and Kinsella (1982) and Utsumi *et al.* (1984), who stated that the interaction between β-7S subunit and B-11S polypeptides at pH 8 and in the presence of 2-mercaptoethanol is mainly electrostatic in nature. Under our experimental conditions, the β-7S/B-11S aggregates would be initially stabilized, at least partially, by hydrophobic interactions. Formation of β-7S/B-11S aggregates, stabilized by SS bonds, was not observed at any of the pHs used in the treatments carried out at 90 °C.

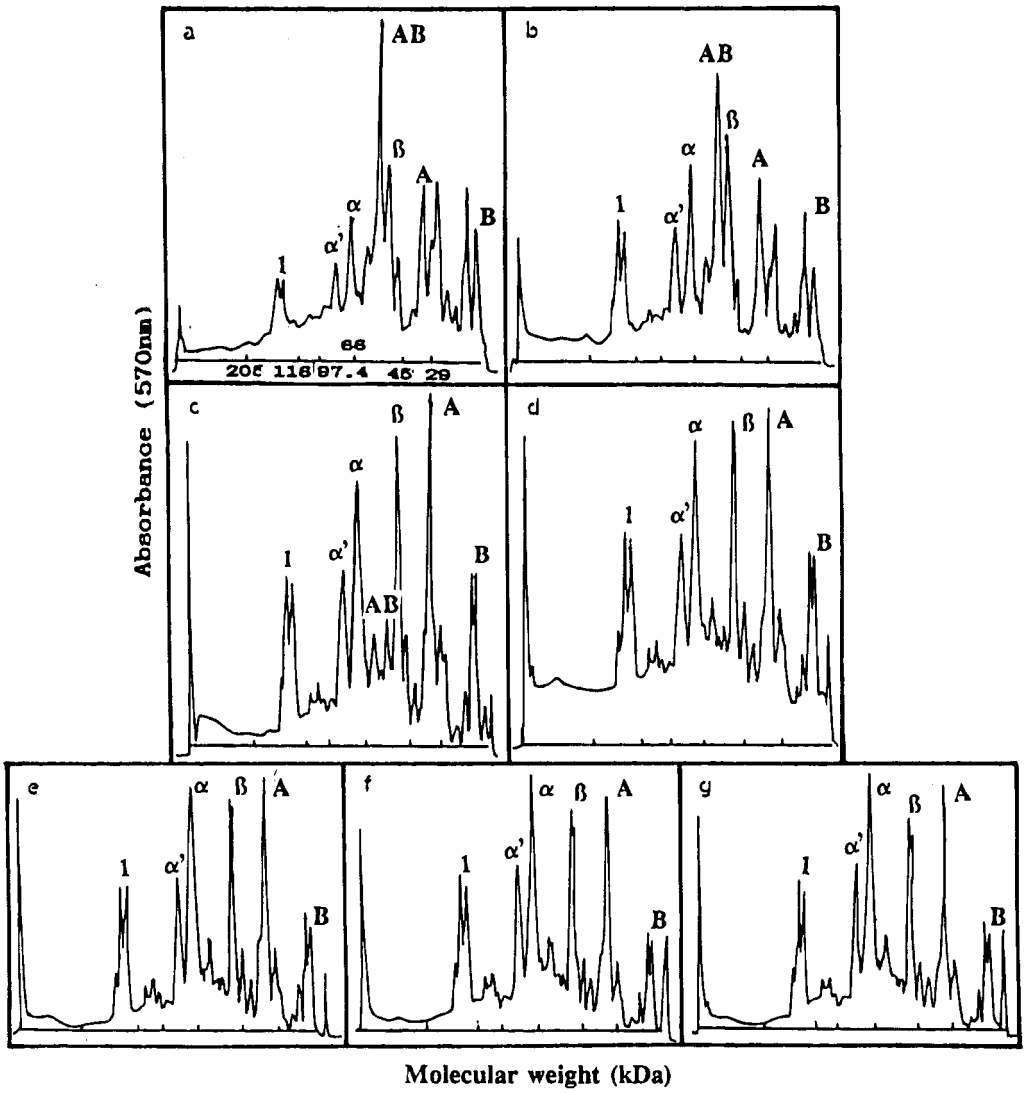

Figure 6. SDS-PAGE densitographies in a 5–15% linear gradient of soy protein isolates (14 mg/mL) treated at 100 °C in the presence of 0.01% Na_2SO_3 for different lengths of time: (a) 0 min, (b) 1 min, (c) 2 min, (d) 3 min, (e) 5 min, (f) 10 min, and (g) 15 min.

Results previously discussed suggest that the pH increase favors denaturating reactions and SH/SS interchange, thereby facilitating dissociation and aggregation reactions. The values of free SH groups (SH_F) corresponding to soy protein isolates treated at different pHs with and without thermal treatment (100 °C, 90 min) are shown in Figure 5. In isolates that did not undergo a thermal treatment, a gradual decrease of the content of free SH groups was observed at pH 7–9; this decrease becoming marked at pH 10. Instead, the values of free SH are much lower after thermal treatment of the isolates, except at pH 10. These results suggest that the thermal treatment favors both the reactions of protein aggregation and oxidation of SH groups. Values of total SH groups (SH_T) show no significant variations (Petruccelli and Añón, 1995).

A thermal-reducing treatment was carried out to determine the influence of Na_2SO_3 on the dissociation–aggregation reactions. The Na_2SO_3 concentration used (0.01%) was calculated to achieve total reduction of the SS bonds present in the soy isolates studied (32 μmol SH/g). Samples (14 mg/mL, pH 7) were treated at 90 and 100 °C for different periods of time. The electrophoretic profile and the variation of the AB-11S/β-7S ratio as a function of the treatment time in isolates treated with Na_2SO_3 are shown in Figures 6 and 7. At zero heating time, the presence of a reducing agent leads to a decrease of the high molecular weight aggregates (100–200 kDa), an increase of the α'-, and α-7S subunits, a slight increase of the A-11S polypeptide, and a decrease of the 29 kDa protein species. The thermal treatment leads to the following: gradual decrease of the AB-11S subunit (this decrease is sharper than that obtained by thermal treatment alone); rupture of the AB-11S subunit, starting at 2 min of heating at 100 °C (this rupture is evidenced by the increase of the band corresponding to the A-11S polypeptide); formation of β-7S/B-11S aggregates, evidenced by a decrease of the band corresponding to the β-7S subunit with regard to the α'- and α-7S polypeptides and by the fact that the B-11S polypeptide shows no increase in relation to A-11S polypeptide; and a decrease to the 29 kDa protein species, which becomes more important if Na_2SO_3 is also present.

These results indicate that the presence of reducing agents in the reaction medium promote the rupture of the AB-11S subunit and the formation of β-7S/B-11S aggregates. The latter result is in agreement with observations of German *et al.* (1982), Damodaran and Kinsella (1982), and Utsumi *et al.* (1984) who stated that β-7S/B-11S interactions, in the presence of 2-mercaptoethanol, are of the electrostatic type and that a

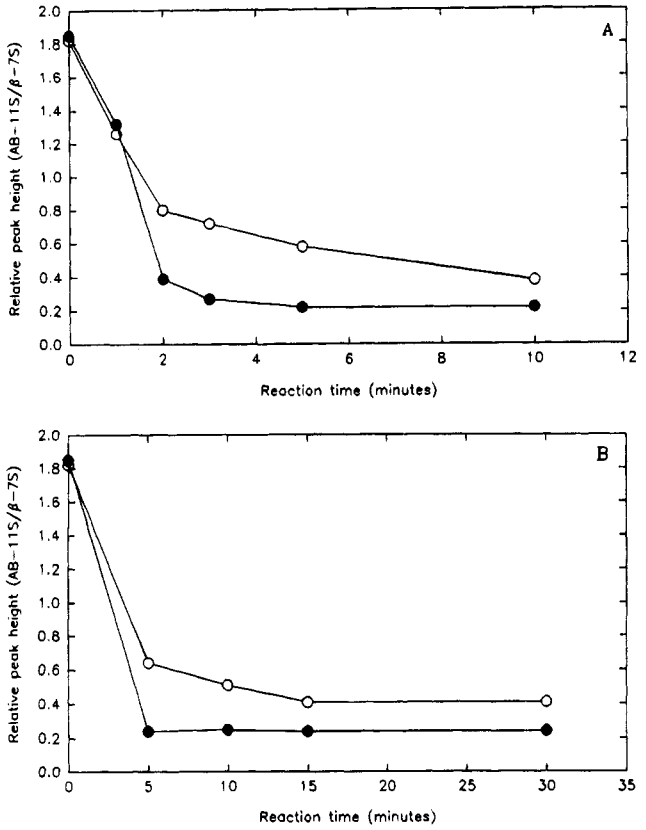

Figure 7. Variation of the peak height ratio of the AB-11S/β-7S aggregate as a function of the heating time at (A) 100 and (B) 90 °C in the presence of 0.01% Na_2SO. Key: (○) control without Na_2SO_3; (●) sample treated with 0.01% Na_2SO_3.

certain conformation of the β-7S subunit is required for them to occur. Moreover, these authors observed that under these treatment conditions, the formation of dimers, trimers, etc. of the B-11S polypeptide are stabilized by SS bonds.

CONCLUSIONS

The results of this study show that the aggregation of AB glycinin subunits of soy protein isolates need thermal treatments of >85 °C. When treatment is carried out at pH 9 or 10, the required thermal treatment time is markedly reduced. Increasing protein isolate concentration enhances aggregation, although it takes place at concentration of <10 mg/mL. The presence of Na_2SO_3 or the pH increase each favor β-7S/B-11S aggregation, probably because they facilitate the dissociation of AB subunits. Aggregation of both AB subunits and β-7S subunit/B-11S polypeptide is due to SH/SS interchange reactions (Mori *et al.*, 1982; Yamagishi *et al.*, 1980, 1981, 1982, 1987). According to our results, β-7S/B-11S aggregation apparently needs conditions other than AB breakdown because with thermal treatment at pH 7 for 20 min, AB dissociation does occur without the formation of β-7S/B-11S aggregates. The interaction between β-7S subunit and the B-11S polypeptides requires an increase of SH groups, which arises when reducing agents are present during thermal treatments or when pH increases. The results also indicate that initially the interaction between the basic polypeptide of 11S and the β subunit of 7S is, at least partially, hydrophobic in nature and later is stabilized by SS bonds. The use of thermal treatments as a denaturating agent to promote the reduction of AB subunits by the action of Na_2SO_3 is not feasible. Denaturating temperatures that allow the Na_2SO_3 to reach the non-exposed bonds result in aggregation, which makes access to bonds difficult.

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