Heat-Induced Interactions between Soybean Proteins: Preferential Association of 11S Basic Subunits and β Subunits of 7S

Shigeru Utsumi, Srinivasan Damodaran, and John E. Kinsella*

Heat-induced interactions between soy 7S and 11S globulins were studied. Heating caused dissociation of both 7S and 11S globulins; the dissociated subunits of 7S and 11S globulins subsequently interacted with each other, forming soluble macrocomplexes having molecular weights over one million. Two-dimensional gel electrophoretic analysis revealed that the macrocomplexes contained predominantly the basic subunits of 11S globulin and the β subunit of 7S globulin; little of the α and α′ subunits of 7S globulin was present in these complexes, indicating that the basic subunits of 11S have higher affinity for the β subunit. The results also indicated that the interaction between the basic subunits and the β subunit is predominantly electrostatic in nature. Furthermore, disulfide bonds between the basic subunits are also involved in the formation of soluble macrocomplexes.

The gel-forming ability of soy proteins is one of the most important functional properties desirable for their usage in conventional foods and as meat extenders. In order to understand the mechanism of gelation of soy proteins, several workers have studied the heat-induced association/dissociation behavior of soy protein isolate and its constituent protein fractions (Mann and Briggs, 1950; Watanabe and Nakayama, 1962; Saio et al., 1968; Wolf and Tamura, 1969; Catsimpoolas et al., 1969, 1970; Aoki, 1970; Fukushima and van Buren, 1970; Hashizume et al., 1975; Hashizume and Watanabe, 1979; Yamagishi et al., 1980; German et al., 1982; Damodaran and Kinsella, 1982). The globulins glycinin (11S) and β-conglycinin (7S) are the major components of soy isolate. The former is composed of six associated dimeric disulfide linked acidic (AS, ~38 000 daltons) and basic subunits (BS, ~20 000 daltons) while the 7S is comprised of at least six combinations of three subunits, denoted α, α′, and β, ranging in molecular weights from 57 000 to 42 000 (Mori et al., 1982b; Thanh and Shibasaki, 1978; Kinsella et al., 1985).

Considerable research has been done on soy 11S globulin with respect to the mechanism of its gelation and the role of the constituent subunits in the formation and properties of the gel (Mori et al., 1982a,b; Nakamura et al., 1983; Utsumi et al., 1983). Furthermore, under identical gelling conditions soy 7S globulin has been shown to possess better gelling properties than soy 11S globulin (Babajimopoulos et al., 1983). Differences in the gelling properties of soy 7S and soy 11S globulins have been attributed to association/dissociation and thermal unfolding characteristics of their constituent subunits (Babajimopoulos et al., 1983). However, it has also been shown that either soy protein isolate or a 1:1 mixture of soy 7S and 11S globulins exhibited better gelling properties than either of the constituent protein fractions (Babajimopoulos et al., 1983). This has been attributed to heat-induced interaction between the constituent subunits of soy 7S and 11S globulins. Recently, we showed that heating of soy isolate at 80 °C in the presence of thiol reagents results in dissociation of both 7S and 11S globulin fractions; the dissociated subunits of 7S globulin subsequently interact electrostatically with the basic subunits of 11S globulin, forming soluble complexes (Damodaran and Kinsella, 1982). Formation of such complexes between the constituent subunits of these major protein fractions may be responsible for the formation of a better three-dimensional network in the gels of soy isolate compared to 11S gels. Although evidence for the heat-induced interaction between soy 7S globulin and the basic subunits of 11S globulin has been presented in the earlier report, the subunit composition and the molecular size of the complexes formed were not analyzed (Damodaran and Kinsella, 1982).

In this paper we report that among the subunits of 7S globulin the β subunit exhibits marked affinity for the basic subunits of 11S globulin. Also, the interaction between the subunits of 7S globulin and the basic subunits of 11S globulin leads to formation of highly heterogeneous soluble macrocomplexes with a minimum molecular weight of about one million.

MATERIALS AND METHODS

Materials. Electrophoretic-grade sodium dodecyl sulfate ($NaDodSO_4$) was purchased from Bio-Rad (Richmond, CA). Acrylamide and bis(acrylamide) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals used in this study were reagent grade.

Isolation of Soybean Proteins. 11S and 7S globulin rich fractions were isolated from defatted, low heat treated soy flour according to the method of Thanh and Shibasaki (1976a,b). The 11S globulin fraction was further purified by Fractogel TSK HW-55 under the conditions described previously (Damodaran and Kinsella, 1981). In the case of the 7S globulin fraction, no further purification was done. The purity of these two protein fractions were >90% and >70%, respectively. Proteins were stored in lyophilized form until used.

Heating Experiments. Heating experiments were carried out in a water bath at 80 °C. In the case of the experiments with mixtures of 7S and 11S globulins, the protein concentration was maintained at 0.5% (1:1 ratio), whereas for experiments with single protein fractions, 0.25% protein concentration was used. All the experiments were done in 30 mM Tris (pH 8.0) containing 10 mM 2-mercaptoethanol (heating buffer) in glass capillary tubes. At the end of the heating period the tubes were removed and immediately cooled with water at 10 °C.

Gel Electrophoresis. One-dimensional polyacrylamide disc gel electrophoresis (PAGE) was performed essentially according to the method of Davis (1964) using 6.5% (w/v) polyacrylamide separating gels in glass tubes (7 × 0.5 cm). In order to separate higher molecular weight protein complexes, two stacking gels (3 and 4% acrylamide) were used in each tube. Protein samples (40 μg) were applied to each gel, and the electrophoresis was performed at 1

Institute of Food Science, Cornell University, Ithaca, New York 14853.

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mA/gel tube for 3 h. Disc gels intended for subsequent second-dimensional electrophoresis were loaded with 250-μg protein samples. In order to prevent leaching of the high molecular weight complexes that did not enter the upper stacking gel, the top of the stacking gel in each tube was polymerized with 4% acrylamide immediately after electrophoresis. The gels were stored in 10% glycerol at −20 °C (Miyazaki et al., 1978) until used for second-dimensional electrophoresis.

Second-dimensional slab NaDodSO$_4$–PAGE was performed according to the method of Laemmli (1970) using 11% (w/v) polyacrylamide gel (14.5 × 14.5 × 0.1 cm). Prior to the second electrophoresis, each first-dimension disc gel was treated with 10 volumes of NaDodSO$_4$ buffer composed of 62.5 mM Tris-HCl (pH 6.8), 1% NaDodSO$_4$, 0.2M 2-mercaptoethanol, and 20% sucrose for 30 min at 55 °C with two changes of solution. The treated gel was placed on top of the second-dimension NaDodSO$_4$ slab gel and then polymerized with 1% agarose solution containing 1% NaDodSO$_4$, 62.5 mM Tris (pH 6.8), 0.2 M 2-mercaptoethanol, and 0.01% bromphenol blue to fix the first-dimension gel. Electrophoresis was carried out at a constant current of 14 mA for 5 h. After electrophoresis the gels were stained with Coomassie Brilliant Blue R250 and scanned with a gel scanner.

Chemical Modification. Citraconylation of 7S globulin was carried out as described by Brinegar and Kinsella (1981). Under the condition employed, the extent of citraconylation was assumed to be 100%. Decitraconylation was carried out by incubating the samples of pH 3.0 in 0.2 M acetate buffer for 2 h at 30 °C (Shetty and Kinsella, 1980).

Sucrose Denisty Gradient Centrifugation. The heat-induced complexes between 7S and 11S globulins were separated by sucrose density gradient centrifugation. The protein sample was loaded on a 10–35% (w/v) linear sucrose density gradient and centrifuged for 16 h at 14 000 rpm in a Sorvall centrifuge (Model OTD-65:B) with a Beckman SW27 rotor. The gradient was then fractionated from the top into 1.0-mL fractions, and the absorbance of each fraction was measured at 280 nm.

RESULTS AND DISCUSSION

In order to study the heat-induced formation of complexes between the 7S and 11S globulin fractions of soy isolate, solutions of 7S (0.25%), 11S (0.25%), and mixtures of 7S and 11S globulin fractions (0.25% of each) were heated at 80 °C for 2 and 30 min. Upon heating, the 11S globulin fraction readily aggregated at 80 °C, whereas the 7S and the 7S + 11S mixture solutions remained clear and showed very little turbidity even after heating for 30 min. The appearance of turbidity in heated glycinin solutions is mainly due to dissociation and subsequent aggregation of the basic subunits of 11S or glycinin (Damodaran and Kinsella, 1982; Mori et al., 1982a). The absence of aggregation of the glycinin fraction in heated soy isolate solutions has been attributed to the formation of soluble complexes between the basic subunits (BS) of glycinin and subunits of β-conglycinin via electrostatic interactions (Damodaran and Kinsella, 1982).

Disc–PAGE analyses were carried out to characterize the molecular size of various soluble protein complexes formed following heating. Preliminary experiments indicated that in the sample containing the heated 7S + 11S mixture a significant amount of the soluble complexes was unable to enter the 4% stacking gel. In order to further characterize these macromolecular complexes, subsequent separations were conducted with disc gels containing two stacking gels composed of 3% and 4% acrylamide. The

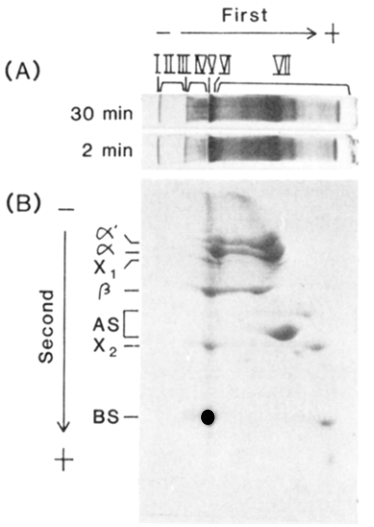

Figure 1. Two-dimensional electrophoresis of the heated 7S globulin fraction. (A) First-dimension disc–PAGE of 7S globulin samples (0.25%) heated at 80 °C for 2 and 30 min. Migration is from left to right. (B) Second-dimension NaDodSO$_4$–PAGE of the disc gel of the 7S globulin sample heated for 2 min. Migration is from top to bottom. The disc gel was divided into regions I–V. Regions I, III, and V represent proteins that could not enter the 3% and 6% stacking gel and the 6.5% separating gel, respectively; II and IV are proteins that stopped in the 3 and 4% stacking gels; VI is the protein located below the top of the separation gel, and VIII is the protein that separated in the separating gel. AS and BS denote the acidic and basic subunits of 11S; α, α', and β are the subunits of 7S and X_1 and X_2 represent two unidentified proteins of molecular weight ~57 000 and ~30 000, respectively.

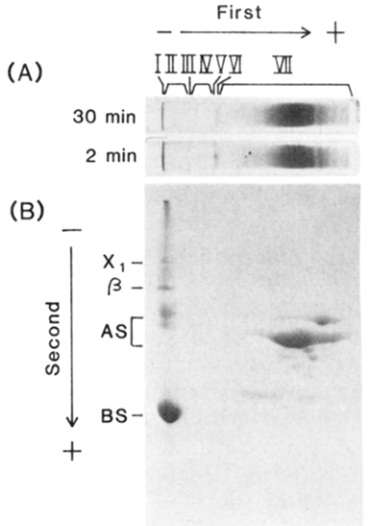

Figure 2. Two-dimensional electrophoresis of the heated 11S globulin fraction. (A) First-dimension disc–PAGE of 11S globulin samples (0.25%) heated at 80 °C for 2 and 30 min. Migration is from left to right. (B) Second-dimension NaDodSO$_4$–PAGE of the disc gel of the 11S globulin sample heated for 2 min. Migration is from top to bottom. See the text for details.

subunit composition of each of the protein bands separated by the first-dimension disc–PAGE was analyzed by a second-dimension NaDodSO$_4$–PAGE and the disc–PAGE patterns of heated solutions of the 7S and 11S globulin fractions, and 7S + 11S mixture are shown in Figures 1–3. For the purpose of identification and characterization of various complexes, the disc gels are divided into various regions, and the protein bands are identified accordingly. Thus, I, III, and V represent the proteins that could not enter the 3% and 4% (stacking gels) and 6.5% (separating)

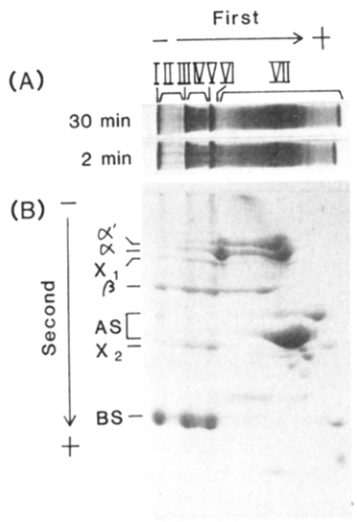

Figure 3. Two-dimensional electrophoresis of the heated 7S + 11S mixture. (A) First-dimension disc–PAGE of the 7S + 11S mixture (1:1) heated at 80 °C for 2 and 30 min. Migration is from left to right. (B) Second-dimension $NaDodSO_4$–PAGE of the disc gel of the 7S + 11S mixture heated for 2 min. Migration is from top to bottom. See the text for details.

gel, respectively; II and IV are the proteins which stopped in 3% and 4% stacking gels; VI is the protein that is located just below the top of the separation gel, and VII corresponds to proteins that are located in the separation gel.

Following heating for 2 and 30 min, the 7S globulin fraction gave similar disc gel patterns, except that the amounts of IV and VI increased and decreased with heating time, respectively (Figure 1). The heated 7S solution contained very little of the proteins corresponding to regions I, II, III, and IV but were rich in protein species corresponding to regions V and VII. Thus, large complexes that did not enter the 3% and 4% stacking gels were not formed in heated 7S solutions. In order to identify the subunit composition of each protein band, a second-dimensional $NaDodSO_4$–PAGE was carried out (Figure 1B). The band corresponding to the V region in the first-dimension predominantly contained the β subunit of 7S globulin and the basic subunits of 11S globulin. The presence of the basic subunit in this band arises mainly from 11S globulin contamination in the 7S globulin sample. Band VI, which is the protein just below the top of the separation gel, contained predominantly the α and the α' subunits of 7S globulin (with $\alpha \gg \alpha'$). The protein bands in region VII of the first-dimensional gel contained α, α', and β subunits of 7S globulin and the acidic subunits of contaminant 11S globulin.

Heating the 11S globulin fraction for 2 and 30 min gave similar disc gel patterns (Figure 2). However, unlike the 7S globulin fraction the 11S globulin fraction showed only protein bands corresponding to regions I and VII of the gel. Band I contained mainly the basic subunits of 11S globulin and the proteins in the region VII contained only the acidic subunits of 11S globulin (Figure 2B); i.e., the precipitate formed during heat treatment of 11S globulin fraction was composed mainly of the basic subunits and the soluble portion contained only the acidic subunits of 11S globulin as reported previously (Damodaran and Kinsella, 1982).

The 7S + 11S mixture (weight ratio 1:1) gave similar disc gel patterns following heating for 2 and 30 min except that the amounts of proteins corresponding to the regions IV and VI increased and decreased, respectively, with heating time (Figure 3). However, unlike the heated 7S and 11S samples (Figures 1 and 2), the heated 7S + 11S mixture contained considerable amounts of proteins corresponding to regions I, III, and V (Figure 3). Since turbidity was not observed in the heated 7S + 11S mixture, these protein bands, which could not enter the 3%, 4%, or 6.5% gels, respectively, represent soluble macromolecular complexes formed following heat treatment at 80 °C. The fact that such complexes were not formed when either the 7S or 11S globulin fraction was heated alone (Figures 1 and 2) strongly suggests that these complexes represent products derived from the interaction between the subunits of 7S and 11S globulins. Analysis of these, i.e., bands I, III, IV, and V, in the second-dimensional $NaDodSO_4$–PAGE clearly indicates that these complexes were composed predominantly of the β subunit of 7S globulin and the basic subunits of 11S globulin (Figure 3). Only small amounts of α and α' subunits of 7S globulin were present in these complexes. Two unidentified proteins with molecular weights of about 57 000 and 30 000 (band X_1 and X_2 in the second-dimension gel) were also associated with the complex. These proteins may be contaminant proteins from the 2S fraction of soy isolate. Most of the α and α' subunits of 7S globulin and the acidic subunits of 11S globulin were present in the VII region of the first-dimension gel, indicating that these exist as monomers in the heated 7S + 11S mixture; i.e., these subunits apparently neither interact with the basic subunits of 11S globulin nor form higher molecular weight complexes via self-association.

The second-dimension pattern of the protein band corresponding to VI of the first dimension revealed that this protein is mainly made up of α and α' subunits (Figures 1 and 3). Bands VI had the same electrophoretic mobility as that of slowly migrating 7S species (data not shown), suggesting that these are isomers of conglycinin. There are seven molecular species ($B_0 \sim B_6$) of 7S globulin with various combinations of the subunits α, α', and β (Thanh and Shibasaki, 1976b; Yamauchi et al., 1981). Some of the 7S species are either made up of three α subunits only (α_3) or a combination of α and α' subunits only (Thanh and Shibasaki, 1976b). The presence of 7S-like species that are solely composted of α and α' subunits in heated 7S and 7S + 11S samples (band VI of Figures 1 and 3) suggests that these species of 7S are either heat stable at 80 °C compared to other 7S species in the native state or are formed during the heating and subsequent cooling processes. Both of these explanations are possible because it has been shown that the α_3 and $\alpha_2\alpha'$ isomers of 7S globulin are present both in the native and in reconsituted 7S samples (Thanh and Shibasaki, 1976b, 1978).

The results (Figure 3) clearly show that, among the three subunits of 7S globulin, the β subunit interacts predominantly with the basic subunits of 11S globulin. This may be due to differences in the physicochemical characteristics of the 7S subunits. Since the interaction between subunits of 7S globulin and the basic subunits of 11S globulin is electrostatic (Damodaran and Kinsella, 1982), the higher affinity of the β subunit for the basic subunit may be due to its higher negative charge density. However, the electrophoretic mobility of the β subunit in the first-dimensional PAGE is less than that of the α and α' subunits (Figure 1), indicating that the β subunit is less negatively charged. This suggests that despite its lower electronegativity, the observed higher affinity of the 7S β subunit for the 11S basic subunit may reflect some unique configuration of the interacting surface. It is conceivable that under the experimental conditions the β subunit may possess a highly localized negative charge that may exhibit stronger electrostatic potential than that of a more diffuse

Table I. Relative Peak Area of the Protein Bands Separated by Two-Dimensional Gel Electrophoresis following Heating of 7S and 11S Soy Proteins[a]

	α'	α	X_1	β	X_2	BS
I	0.07	0.08	0.25	1.69	0.30	9.63
	(0.04)	(0.05)	(0.15)	(1)	(0.18)	(5.70)
III	0.32	0.45	0.68	2.14	1.16	10.68
	(0.15)	(0.21)	(0.32)	(1)	(0.54)	(4.99)
V	0.49	0.89	0.87	2.35	1.53	11.47
	(0.21)	(0.38)	(0.37)	(1)	(0.65)	(4.88)

[a] Each value was calculated from the peak area of the densitogram of Figure 3. The numbers in parentheses are the ratios, taking the β subunit as unity.

charged surface on the α and α' subunits. Furthermore, if the charged surface on the β subunit is located in a partial nonpolar (low dielectric) environment, this would not only enhance the electrostatic potential but also stabilize the electrostatic bond formed between the β subunit and the basic subunit of the 11S.

In order to quantify the amounts of various subunits involved in the formation of soluble macrocomplexes, the protein bands in the second-dimension gel corresponding to bands I, III, and V of the first-dimension PAGE (Figure 3) were scanned with a densitometer, and the results are summarized in Table I. It may be noted that the largest macrocomplex corresponding to band I of the first-dimension PAGE is composed mainly of the β subunit and the basic subunits and very little of other proteins. However, the smaller complexes (bands III and V) contained relatively larger proportions of α, α, α', X_1, and X_2 proteins.

Previously it was suggested that the interaction between the subunits of 7S globulin and the basic subunits of 11S globulin is mainly electrostatic in nature and that the complexes can be dissociated at an ionic strength of 0.4 (Damodaran and Kinsella, 1982). The dissociation of the complex between 0 and 0.1 ionic strength was more pronounced than between 0.1 and 0.4. The ionic strength of the stacking gel in the first-dimension gel is about 0.12. This raises the question as to whether, during electrophoresis in the first dimension, the soluble macrocomplexes undergo dissociation under the prevailing ionic strength conditions. It may be possible that some of the α and α' subunits in the VII region of the first-dimension PAGE (Figure 3) may have originally been associated with the macromolecular complexes (bands I, III, or V of Figure 3) but undergo dissociation during the first-dimension electrophoresis because of the ionic strength conditions in the gel. The following approach was employed to test this possibility: The macromolecular complexes from the heat-treated 7S + 11S samples were separated by centrifugation on a 10–35% linear sucrose density gradient. The major purpose of this step is to remove the uncomplexed subunits of 7S globulin and the acidic subunits of 11S globulin from the macrocomplexes. Under these conditions PAGE analysis of the macrocomplexes should show bands I, III, or V only and no bands corresponding to the low molecular weight subunits of 7S globulin or the acidic subunits of 11S globulin. However, if the macrocomplexes undergo dissociation under the ionic strength conditions of the stacking gel, one should be able to see lower molecular weight bands in the separation gel.

The sucrose density gradient centrifugation profile exhibited a diffuse pattern instead of distinct peaks (Figure 4), indicating that the molecular weights of the complexes were very heterogeneous. The protein complexes contained in fractions 10 and 16 (of Figure 4) were selected for two-dimensional electrophoretic analysis. The first-

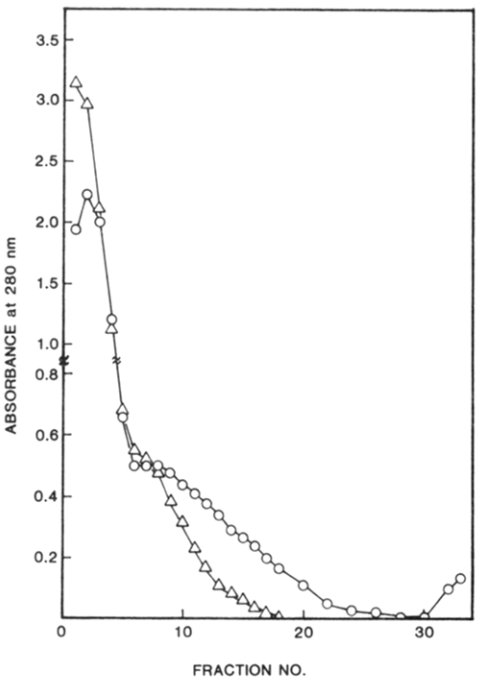

Figure 4. Sucrose density gradient (10–35% w/v) centrifugation of heated 7S + 11S globulin mixtures: (○) native 7S + native 11S mixture heated at 80 °C for 2 min; (△) citraconylated 7S + native 11S mixture heated at 80 °C for 2 min. Sedimentation is from left to right. See the text for details.

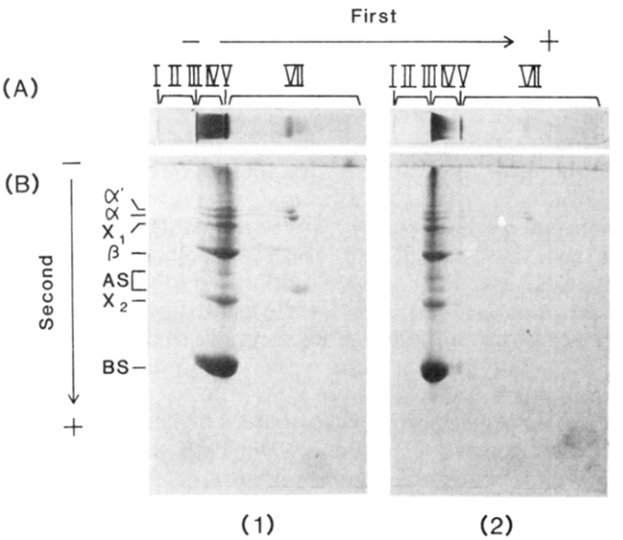

Figure 5. Two-dimensional electrophoresis of the macromolecular complexes separated by sucrose density gradient centrifugation: (1) fraction 10; (2) fraction 16 of the heat-treated native 7S + native 11S sample (see Figure 4). (A) First-dimension disc–PAGE. Migration is from left to right. (B) Second-dimension NaDodSO$_4$–PAGE. Migration is from top to bottom. See the text for details.

dimensional PAGE patterns indicate that fraction 10 is rich in macrocomplexes corresponding to IV and V and a small amount of III, whereas fraction 16 is rich in III and only a small amount of V. However, both PAGE patterns exhibit a small molecular weight band in the separation gel (Figure 5). Since the mobility of this band is the same as that of the 7S subunits (M_r 60 000), it is improbable that this protein is an overlapping contaminant. However, it is conceivable that this protein band results from the dissociation of some of the subunit components of the macromolecular complexes at the ionic strength conditions of the stacking gels. Analysis of these PAGE gels by second-dimensional SDS–PAGE revealed that the ma-

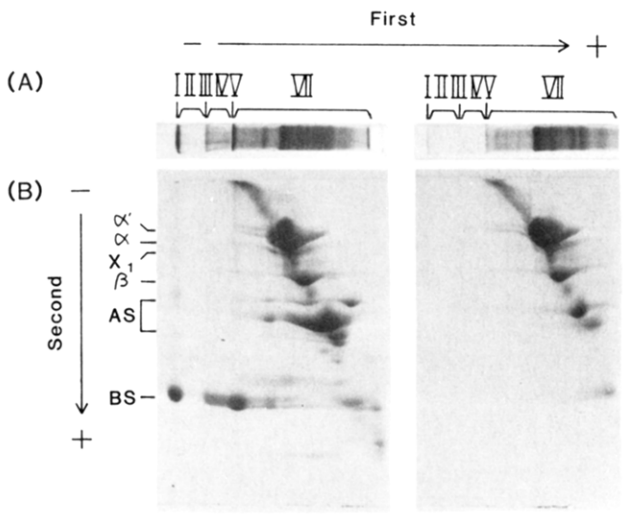

Figure 6. Two-dimensional electrophoresis of the heated mixture of citraconylated 7S + native 11S (1) and citraconylated 7S globulin fraction (2). (A) First-dimension disc–PAGE. (B) Second-dimension $NaDodSO_4$–PAGE. The experimental conditions were as described for Figures 1–3.

crocomplexes contain mainly α, α', X_1, β, X_2, and the basic subunits of 11S. The concentration of the β subunit in these macrocomplexes is greater than those of the α and α' subunits, and the low molecular weight protein in band VII (PAGE, Figure 5) contains solely the α and α' subunits. This suggests that under the ionic strength conditions of the gel, the two electrostatically bound complexes composed of an α basic subunit and α' basic subunit undergo dissociation, the extent of dissociation depending upon the equilibrium constant prevailing at the ionic strength in the stacking gel. However, the electrostatic bonds between the β subunit of 7S and the basic subunits of 11S in the complex are apparently stronger and remain intact under the ionic conditions of the gel. These results demonstrate that at least three macrocomplexes are formed on heating 7S and 11S but two of them undergo dissociation during electrophoresis. Apparently the affinity of the β subunit for the basic subunit is much stronger than that of the α and α' subunits, and thus it remains intact during electrophoresis.

As mentioned earlier, the apparent higher affinity of the β subunit of 7S globulin for the basic subunit of 11S globulin may be due to a certain unique configuration of the interacting site that provides a partial nonpolar environment and thus stabilizes the electrostatic bonds. Any change in this configuration could decrease the affinity of the β subunit for the basic subunits. In order to assess this, heat-induced interactions between citraconylated 7S globulin and native 11S globulin were studied. The rationale behind this approach is that citraconylation of 7S globulin increases the electronegativity of α, α', and β subunits alike and induces conformational changes in the β subunit, thus altering the specific configuration of the interacting site(s) on this subunit.

The two-dimensional electrophoretic pattern of the heat-treated citraconylated 7S + 11S sample is shown in Figure 6A. Heat-treated citraconylated 7S + native 11S samples were electrophoresed directly on the first-dimensional PAGE; in order to decitraconylate the proteins in the gel, the first-dimension gel was incubated in 0.2 M acetate buffer at pH 3.0 for 2 h at 30 °C with two changes of the buffer. Then the gel was incubated in the $NaDodSO_4$ buffer (see Materials and Methods) before carrying out the second-dimension electrophoresis (Figure 6). There is a striking difference between the two-dimensional electrophoretic patterns of the complexes formed between native 7S + 11S (Figures 3 and 5) and the citraconylated 7S + 11S (Figure 6A). The complexes, I, III, and V of the former show distinct bands of α, α', and β in the second dimension while the latter seems to contain only the basic subunits of 11S globulin. Since no turbidity was observed during heating of the citraconylated 7S + 11S mixture, these bands cannot be attributed to aggregated basic subunits. This explicitly suggests that there is indeed formation of soluble complexes between the basic subunits of 11S globulin and the citraconylated subunits of 7S globulin as in the case of the native 7S globulin. But the absence of α, α', and β subunit bands in the second dimension of these complexes (Figure 6A) may be attributed to dissociation of these complexes under the ionic strength conditions of the first-dimension stacking gel (Figure 6A). In other words, the macromolecular complexes formed between the citraconylated α, α', and β subunits of 7S globulin and the basic subunits of 11S globulin are highly unstable under the ionic strength conditions of the gel and hence dissociate during electrophoresis. While the dissociated α, α', and β subunits migrate into the separation gel, the dissociated basic subsunits spontaneously aggregate via hydrophobic interactions (German et al., 1982) at the pH and ionic strength conditions of the first-dimension gel.

Comparison of Figures 3 and 6A suggests that although citraconylated α, α', and β subunits are highly negatively charged, the electrostatic bonds formed between these modified 7S subunits and the basic subunits are weaker compared to those between the native subunits and are completely neutralized at 0.12 ionic strength of the stacking gel. These results confirm our notion that the observed higher affinity of the β subunit for the basic subunits is related not only to its electronegativity but also to the unique configuration of the interacting site in the native β subunit. Citraconylation results in destabilization of this configuration and hence decreases the affinity of the β subunit for the basic subunits.

The sucrose density gradient sedimentation behavior of the complexes formed between the citraconylated 7S + native 11S mixture is shown in Figure 4. Even in this case the gradient profile exhibited a diffused pattern, indicating that the molecular sizes of the complexes are highly heterogeneous. Furthermore, examinatiion of the curves in Figure 4 clearly suggests that the interaction between native 7S and 11S globulins favor formation of higher molecular weight complexes compared to those of the citraconylated 7S globulin. Although electrostatic interactions play a major role in the formation of complexes of both cases, some other type of interactions in the complexes, which are characteristic of the native 7S subunits, seem to favor formation of larger soluble complexes.

The fact that the soluble complexes do not enter the 3% and 4% stacking gels suggests that the minimum molecular weight of these complexes is more than one million. Since there are free sulfhydryl groups in the dissociated basic subunits, it is probable that the three-dimensional network of these complexes may contain disulfide bonds between the basic subunits in the complex. In order to confirm the existence of such disulfide linkages, the macrocomplexes I and III of the first-dimensional PAGE (Figure 3) were analyzed as follows: bands I and III were cut from the first-dimensional gel and electrophoresed separately in the second-dimensional $NaDodSO_4$–polyacrylamide disc gel without treatment with 2-mercaptoethanol. These second dimension gels were than soaked in the second-dimensional $NaDodSO_4$ buffer containing 0.2 M 2-mercaptoethanol and

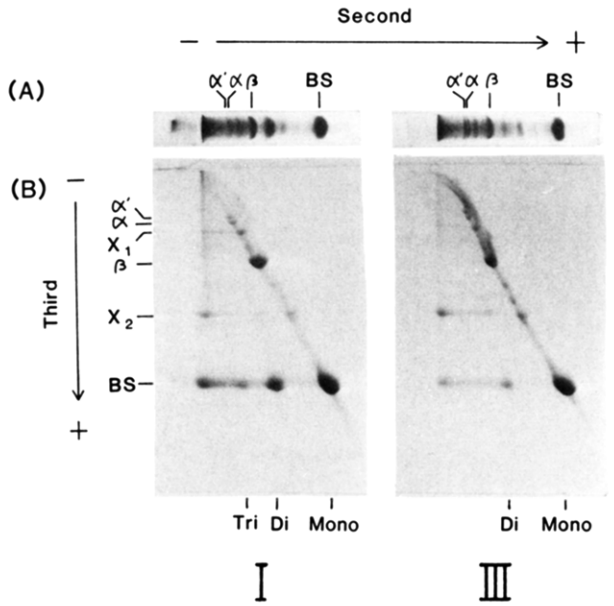

Figure 7. Three-dimensional electrophoresis of the heated mixture of native 7S + native 11S globulins. (A) The bands corresponding to I and III of the first-dimension PAGE (Figure 3) were cut out and treated with $NaDodSO_4$ buffer containing no 2-mercaptoethanol and then electrophoresed on disc–$NaDodSO_4$–PAGE (second dimension). (B) The second-dimension disc gels were then treated with $NaDodSO_4$ buffer containing 0.2 M 2-mercaptoethanol and electrophoresed on a slab $NaDodSO_4$–PAGE as described in Figure 3 (third dimension). See the text for details.

then relectrophoresed on a third-dimensional $NaDodSO_4$–polyacrylamide slab gel. The rationale for this approach was that if disulfide bonds are not present in the network of the complexes, then all the bands in the third-dimension gel should lie on an oblique diagonal line. On the other hand, if disulfide bonds are present in these complexes, then the third-dimension gel should exhibit certain protein bands that were originally held together by disulfide bonds in the second-dimensional gel below the olique line. The results of such an analysis of the macrocomplexes I and III are shown in Figure 7, which clearly demonstrate the presence of disulfide bonds in the macrocomplexes. Since most of the bands below the oblique line correspond to the basic subunits, the disulfide bonds in the macrocomplexes exist only between the basic subunits of 11S and not between the basic subunits and α, α', or β subunits of 7S globulin. The results also suggest that most of the basic subunits in the complex exist as monomers interacting electrostatically with the subunits of 7S globulin; considerable amounts of the basic subunits also exist as dimers or trimers linked by disulfide bonds (Figure 7A).

The results presented here clearly demonstrate that thermal treatment of soy 7S + 11S mixtures induces formation of soluble macrocomplexes between the subunits of 7S globulin and the basic subunits of 11S globulin. Among the subunits of 7S globulin, the β subunit exhibits remarkable affinity for the basic subunits. About 80% of the 7S subunits associated with the macrocomplexes in the β subunit, and most of the α and α' subunits remain as uncomplexed monomers in the heated solution. This is in contrast to our previous speculation that all the subunits of 7S globulin were involved in the formation of soluble complexes with the basic subunits.

Although the results suggest that the macrocomplexes are made up of mainly the β subunit and the basic subunits, the molecular size and subunit composition of these

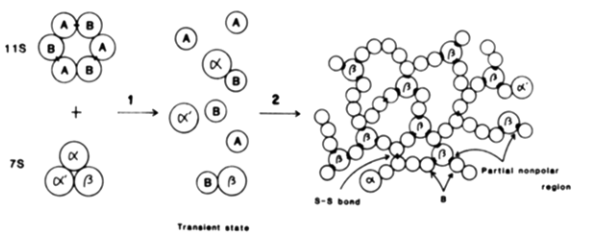

Figure 8. Schematic representation of the heat-induced formation of soluble macromolecular complexes between the subunits of 7S and 11S globulins. α, α', and β refer to the three subunits of 7S globulin and A and B refer to the acidic and basic subunits of 11S globulin. These are mostly linked by one intermolecular disulfide (SS) as depicted by the black line linking the A and B subunits in the half 11S molecular shown. Reaction 1 represents the dissociation induced by heating at >80 °C in the presence of thiol (RSH) reagent and the initial electrostatic association of the heat-dissociated 7S subunits with the heat plus RSH-dissociated basic (B) subunits of 11S. The dissociated acidic subunits remain as monomers in solution. Further heating (2) results in gradual polymerization to a soluble macromolecular complex composed mostly of β subunits of 7S associated electrostatically with BS of 11S at contact points that are relatively nonpolar. A few α and α' subunits and some disulfide-linked basic subunits occur in the complex. (Note: subunits are not drawn to scale.)

complexes are highly heterogeneous. This poses difficulty in calculating the mole ratio of each subunit in these complexes. However, on the basis of the results presented here, the various molecular changes in soy isolate during thermal treatment may be schematically represented as shown in Figure 8. Since such changes and formation of macrocomplexes may occur during the conditions of thermal gelation of soy proteins, further understanding of the physicochemical nature of these complexes may provide an insight on the mechanism of thermal gelation as well as rheological properties of soy gels.

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Studies on the Proteins from Safflower Seed (*Carthamus tinctorius* L)

T. S. Latha and V. Prakash*

Safflower seed (*Carthamus tinctorius*) was analyzed for moisture, protein, ether extractives, ash, total sugars, and crude fiber. The total protein in the seed was extracted at pH 7.5 by low ionic strength buffers in the presence of sodium chloride and at different pHs. The extracted total protein was analyzed by using the techniques of sedimentation velocity, polyacrylamide gel electrophoresis, ion-exchange chromatography, and gel filtration. It consists of predominantly one major fraction of sedimentation value 12S and three other components of sedimentation values 2S, 7S, and 17S. The protein has an absorption coefficient, $E^{1\%,1cm}_{280nm}$, of 17.3 and an absorption maximum at 279–280 nm in the ultraviolet region. The presence or absence of sodium chloride during extraction has a profound effect on the extractability of the 12S component of the total protein.

Safflower (*Carthamus tinctorius* L.) seed is primarily grown for its oil. The seed contains nearly 35–40% oil, 15–20% protein, and 35–45% hull fraction (Betschart et al., 1975). The proteins from the safflower seeds are of good nutritional quality (Betschart et al., 1979; Kohler et al., 1966). The ultilization of the safflower proteins and protein isolates for food and feed purposes has been attempted (Betschart et al., 1975). However, utilization of safflower protein concentrate in food has been limited, because of color and bitter principles and also the high content of crude fiber (Lyon et al., 1979). Attempts have been made to obtain a protein concentrate low in crude fiber content (Betschart et al., 1975; Kohler, 1966).

However, much information is not available on the nature of the proteins of safflower. In the present investigation, total proteins have been isolated and analyzed by using various physicochemical techniques.

MATERIALS AND METHODS

Preparation of Defatted Safflower Meal. Safflower seeds of the variety A-1 were obtained from the Karnataka State Seed Corp., Ltd., Bangalore, India. They were flaked and dried in a cabinet air dryer at 60 °C for 3 h. The dried flakes were defatted with *n*-hexane and were air-dried to remove the residual solvent. The hulls were separated by differential sieving. The hull-free meal was passed through a plate mill and then passed through a 60-mesh sieve. The resultant flour had less than 1% fat.

Proximate Composition. Moisture, protein ($N \times 6.25$), ether extractives, ash, and crude fiber were determined by the AOAC (1980) method. Total sugars were determined by the phenol–sulfuric acid procedure (Montgomery, 1961).

Nitrogen Extractability. Two grams of the safflower flour was mixed with 20 mL of the solvent; the pH was adjusted to the desired value by 5 N HCl or 5 N NaOH. The suspension was shaken in a rotary shaker for 1 h at room temperature (~27 °C). The slurry was centrifuged at 6000*g* for 20 min in a Sorvall RC-5B refrigerated centrifuged at 20 °C. The pH of the supernatant was read in a Toshniwal pH meter, type CL 41. Aliquots (5 mL) were used for nitrogen determination by the Kjeldahl method (AOAC, 1980). The influence of parameters such as extraction time, solute to solvent ratio, pH, and various solvents for extraction of the protein was investigated by using the defatted flour.

For extractability studies the suspension pH was measured at intervals of 5 min after the slurry is made, and the pH was adjusted to a constant value within the first 15–20 min. The magnitude of the drift was of the order of 0.3 unit at extreme pH values and about 0.1 unit at neutral pH values. The pH of the slurry after the extraction time, i.e., 1 h was again measured. This pH value was used for plotting the data.

Extraction of Total Proteins. The total proteins from the safflower meal were extracted in 0.01 M phosphate buffer of pH 7.5, containing 1 M NaCl (hereafter referred to as PS buffer), by using a meal concentration of 10%. The slurry was centrifuged at 6000*g* at 20 °C. The supernatant was dialyzed against the same buffer and used for various experimentations after determining the concentration.

Protein Concentration. The protein concentration was routinely determined by using a value of $E^{1\%,1cm}_{280nm} = 17.3$ for the total protein. This was determined by measuring the absorbance of protein solutions of known concentration.

Gel Filtration. Sepharose 6B-100 (medium) gel in PS buffer was packed into a column, 1.8 × 100 cm. Approximately 40 mg of the protein in the above buffer was loaded onto the column and was eluted with the same buffer. Fractions (2.5 mL) were collected with an Emenvee au-

Protein Technology Discipline, Central Food Technological Research Institute, Mysore 570013, India.

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