Thermal Dissociation and Association Behavior of Soy Proteins

Bruce German, Srinivasan Damodaran, and John E. Kinsella*

Solutions of soy proteins or glycinin (11 S) plus conglycinin (7 S) do not aggregate when heated at 100 °C at neutral pH for 30 min. Thermal studies of these proteins using differential scanning calorimetry showed thermal transitions occurring at 92 and 77 °C for 11 S and 7 S, respectively. At temperatures in excess of 90 °C, isolated glycinin (11 S) forms insoluble aggregates that consist exclusively of the basic subunits. Response surface analyses of the solubility of isolated basic subunits indicate that the basic subunit peptides spontaneously coagulate via hydrophobic interactions. The absence of aggregation in whole soy mixtures suggests an interaction between 11 S and 7 S following heating, resulting in either stabilization of the 11S structure or solubilization of the component basic subunits following dissociation. Reversible cross-linking experiments suggested that heating 11 S in the presence of 7 S results in complex formation between dissociated 7S subunits and 11S basic subunits.

The thermal stability of food proteins functionally connotes their resistance to aggregation in response to heating (Kinsella, 1979). Heat-induced gelation is a requirement in such products as sausage and cheese analogues in which thermal gelation is necessary for structure and emulsion stability, and in egg white based cakes heat setting of the foam batter without collapse is critically important. In the selection of substitute proteins for these products equivalent thermal characteristics are required. However, in the case of soy proteins, one of the factors limiting their utilization as a highly functional food protein is their rather high stability to heat. This has generally been interpreted to be the result of extensive disulfide bridging within the major globulin protein, 11 S (Kinsella, 1979). However, under conditions favoring thermal dissociation and aggregation of isolated 11 S, the thermal aggregation of whole soy protein mixtures, e.g., soy isolate, is minimal. This suggests that interactions between different globulins in whole soy proteins affect the net thermal properties, i.e., impart stability.

Gelation is generally exhibited by soy proteins at relatively high concentrations (Hermansson, 1979), which makes analysis of molecular interactions difficult. In addition, because of the complex oligomeric nature of the two major soy fractions, effective elucidation of the behavior of these proteins in response to heating requires analysis of the thermal and aggregation properties of each of the subunit peptides separately and the characterization of critical interactions between them. We have studied the dissociation of the quaternary structure of 11 S and the subsequent association of the peptides released.

The heat denaturation of simple monomeric proteins in isolated systems has been successfully explored and characterized thermodynamically by using scanning calorimetry (Privalov, 1979), spectroscopy (Hermans and Scheraga, 1961), and magnetic resonance (Matthews and Westmoreland, 1975). Denaturation is closely modeled by a two-state cooperative transition confined to a narrow temperature range. Oligomeric proteins exhibit additional complexities due to the release of several protomers previously associated. This produces two essentially independent thermal events: first, disruption of the oligomeric structure that itself can result in aggregations and, second, denaturation of the actual monomers initiating further rearrangements and/or aggregations. Soy proteins constitute such a system, and while thermal transitions below 100 °C have been characterized (Hermansson, 1979; Lewis and Chen, 1979), still much is unknown about the specific alterations in the quaternary and secondary structure of these proteins.

Catsimpoolas (1969) and Wolf and Tamura (1969) showed that isolated 11S protein, the major constituent of soy, underwent profound structural changes when heated. Significantly, when heated at low ionic strength and in the presence of reductant, the protein readily dissociated into subunits and aggregated. Both the rate and extent of aggregation were enhanced by the presence of reductant (Catsimpoolas et al., 1970). This indicated that the 11S oligomeric structure was stabilized by disulfide bridges.

Additionally, these authors found that the extent of aggregation decreased dramatically on increasing ionic strength. This suggested that the oligomeric structure is destabilized by structure-specific electrostatic repulsions. Neutralization of these charged groups by the addition of salt should therefore stabilize the structure. This would predict an increase in the thermal transition temperature of 11 S in the presence of salt, which has in fact been shown by Hermansson (1979). Badley et al. (1975) and Kitamura et al. (1976) reconciled much of these results in terms of a model of 11 S composed of pairs of acidic and basic subunits joined into intermediary subunits by one or more disulfide linkages. Cleavage of the disulfide bonds therefore permits dissociation of the intermediate subunits in response to heating, which allows aggregation and precipitation of the dissociated peptides.

In subsequent studies under similar reducing conditions but in the presence of the 7S protein, in spite of extensive alterations in the conformations and ultracentrifugal patterns of the proteins no precipitation was observed (Hashizume and Watanabe, 1979). However, the addition of salt to these whole soy mixtures induced aggregation. These observations suggest an interaction between the 7S and 11S proteins, which may to some extent account for the heat stability of the whole soy proteins. The studies reported here were conducted to investigate the physicochemical basis of the stabilization of soy 11 S by 7 S.

MATERIALS AND METHODS

Fractionation of Soy Globulins. Soy 7 S and 11 S were isolated by using the method of Thanh and Shibasaki (1976), based on the differing solubilities of the proteins in Tris buffer at 4 °C. Purity of the fractions was determined by electrophoresis as described below.

Electrophoresis. The protein subunits were separated electrophoretically under denaturing and reducing conditions. Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was carried out by using a

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

0021-8561/82/1430-0807$01.25/0

modification of the method of Laemmli (1970). Separating slab gels consisted of linear gradients of 5–20% acrylamide. Samples of 0.1–0.2% protein were made up in 10 mM Tris-HCl dissociating buffer, pH 6.8, containing 8 M urea, 2% $NaDodSO_4$, 1% 2-mercaptoethanol, and 0.025% bromphenol blue. Samples were loaded at 20 μL/channel and run at 14-mA constant current for 4 h. Gels were fixed and destained in 20:10:70 methanol–acetic acid–water and stained in the same fixer with 0.05% Coomassie blue R. The gels were scanned in an E-C densitometer (St. Petersburg, FL) interfaced to a Spectra-Physics (Santa Clara, CA) computing integrator.

Isolation of 11S Subunits. Acidic and basic subunits were isolated to investigate the forces leading to the aggregation of these subunits upon dissociation. Isolation was performed by using ion exchange of dissociated subunits after the method of Catsimpoolas et al. (1971). Fractionated 11 S was dissociated in 0.05 M phosphate buffer (pH 7.6) containing urea (6 M) and dithiothreitol (1 mM; DTT). This solution was overlayed onto a funnel containing DEAE-Sephadex preequilibrated with the buffer. The unbound protein (basic subunits) was washed out until absorbance at 280 nm reached 0. The Sephadex was then eluted with the same buffer containing 0.1 M NaCl and the eluent (acidic subunits) collected until absorbance reached 0. The basic subunits were dialyzed at pH 3.0, the acidic subunits were dialyzed at pH 8.0, and they were used immediately for analyses. Purity was determined electrophoretically in $NaDodSO_4$ gels.

Solubility of 11S Subunits. pH solubility profiles were generated by monitoring turbidity and measurement of solubility estimated by absorbance at 280 nm after centrifugation of heated dispersions of the proteins. Turbidity measurements were carried out by using 0.1% protein solutions made up to a specific pH. Solubility studies were performed as follows: 0.1% protein solutions were adjusted to specified pH, salt, and DTT concentration, stirred for 10 min, and centrifuged at 20000 *g* for 15 min. The absorbances of the supernatants were measured at 280 nm. Data were generated according to a central composite design (Cochran and Cox, 1957) by using ionic strength, DTT concentration, and pH as independent variables. Solubilities were fitted by least squares to response surfaces with MINITAB (Penn State).

Differential Scanning Calorimetry. Differential calorimetry was used to establish whether the presence of 7 S could affect the thermal quaternary stability of 11 S and therefore alter the measurable thermal transition temperature of the 11S protein. Thermoprofiles were determined by using a Perkin-Elmer DSC 2 differential scanning calorimeter. Protein solutions (8%) in 10 mM Tris buffer at pH 8.0 were scanned at 5, 10, 20, and 40 K/min to determine temperatures of transition. The least-squares method was used to extrapolate the temperature of transition at zero scanning rate.

Reversible Protein Cross-Linking. For isolation of a complex of 7S and 11S subunits, the protein species remaining after heating were covalently cross-linked. Solutions consisting of 7 S (0.05%) and 11 S (0.05%) were heated to 90 °C for 30 min in Tris buffer containing 10 mM 2-mercaptoethanol and then cooled. Free thiol groups were blocked by using sodium tetrathionate and then dithiobis(propionimidate), a cleavable amine cross-linking agent (Sigma), was added to a final concentration of 3 mg/mL. This reagent reacts specifically with primary amino groups to form an amidine linkage (Peters and Richards, 1977). The solutions were mixed, incubated for 1 h, and quenched with excess ammonium acetate. The proteins were precipitated with acetone and made to 0.5% concentration in Tris buffer, pH 6.8, containing 8 M urea and 2% $NaDodSO_4$. Samples were separated electrophoretically, stained, and scanned as above. Single channels containing separated proteins were then cut from the gel and incubated in dissociating buffer containing DTT (10 mM) for 15 min. These gel slices were then placed on a second slab gel and electrophoresed as previously described. After separation the resulting gel was stained and scanned as described above.

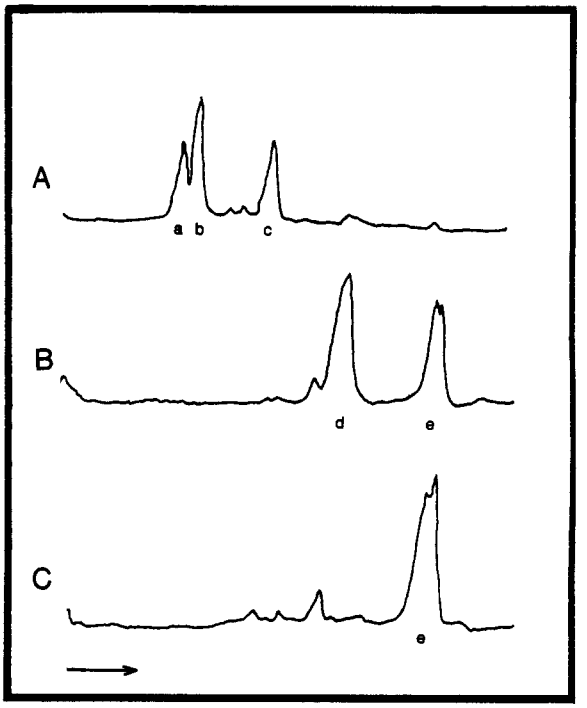

Figure 1. Densitometer scans of $NaDodSO_4$–polyacrylamide gels under dissociating (8 M urea) and reducing (1 mM DTT) conditions. (A) Soy 7 S: (a) molecular weight band corresponding to the α′ subunit, (b) the α subunit, and (c) the β subunit. (B) Soy 11 S: (d) acidic subunits, (e) basic subunits, and (c) the precipitate after heating 11 S alone to 90 °C for 30 min in Tris buffer containing 10 mM 2-mercaptoethanol.

RESULTS AND DISCUSSION

Isolation of Soy 11 S and 7 S. Both 11 S and 7 S are made up of several subunits (Catsimpoolas, 1969; Thanh and Shibasaki, 1979). $NaDodSO_4$–polyacrylamide gel electrophoresis under denaturing and reducing conditions readily separated the two classes of subunits that make up the 11S protein and the three classes of subunits comprising the 7S protein. Typical densitometer scans showing the separation of these subunits by electrophoresis are given in Figure 1. The 7S proteins readily dissociated in $NaDodSO_4$ into the three possible subunits as described by Thanh and Shibasaki (1976). Separation of the 11S protein subunits requires in addition to $NaDodSO_4$ a reductant, e.g., DTT (1 mM), to resolve two molecular weight classes of subunits. These are the acidic and basic subunits with molecular weights of approximately 36000 and 18000, respectively (Catsimpoolas, 1969).

Thermal Precipitation of Soy 11 S. In Tris buffer, pH 8.0, containing 2-mercaptoethanol (10 mM), the isolated 11S protein readily forms a colloidal precipitate when heated at 90 °C (Wolf and Tamura, 1969). The electrophoretic pattern of this precipitate is shown in Figure 1. Clearly turbidity under these conditions can be ascribed entirely to the aggregation of the basic subunits. The acidic subunits remain soluble at this pH in spite of dis-

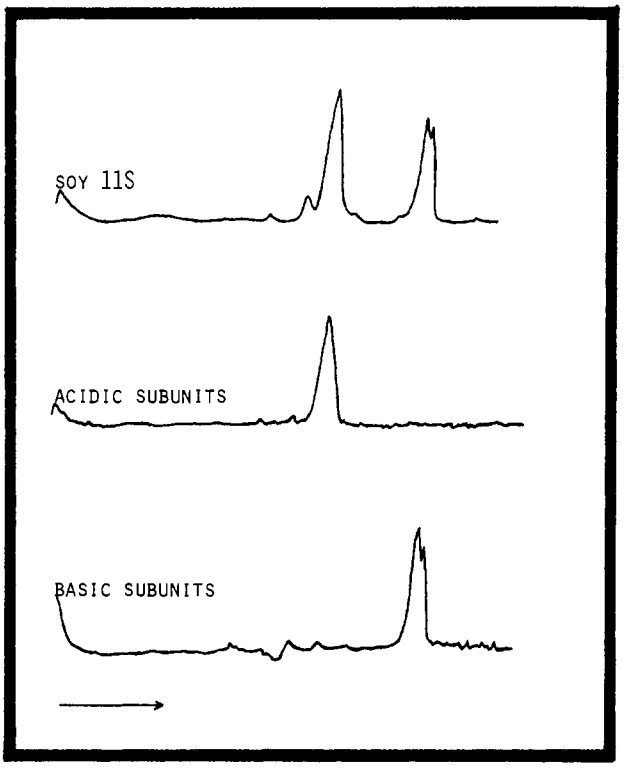

Figure 2. Densitometer scans of $NaDodSO_4$–polyacylamide gels of soy 11S and isolated subunits, run under dissociating (8 M urea) and reducing (1 mM DTT) conditions.

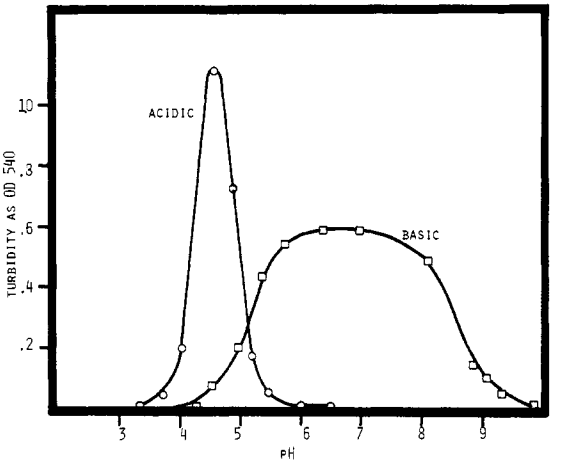

Figure 3. pH solubility profile of 11S subunits as turbidity at 540 nm: 0.1% solutions of (○) acidic subunits and (□) basic subunits.

sociation from the oligomer.

Separation of 11S Subunits. For examination of the mechanism of aggregation the 11S subunits were isolated by using urea and DTT dissociation and subsequent separation using ion-exchange resin in quantities sufficient for gross solubility determinations. Kamata et al. (1979), using fluorescence, ORD, and CD as structural probes, have reported that under these conditions the peptides regain virtually all their native secondary structure. This suggests that while the subunits are dissociated they are otherwise minimally altered. Typical densitometric scans of gels of the separated subunits are shown in Figure 2.

The pH profiles of the proteins were measured by using turbidity as an estimate of insolubility (Figure 3). The acidic subunits were characterized by a narrow solubility profile typical for highly acidic proteins with a large surface charge density titrating over a narrow pH range. The basic

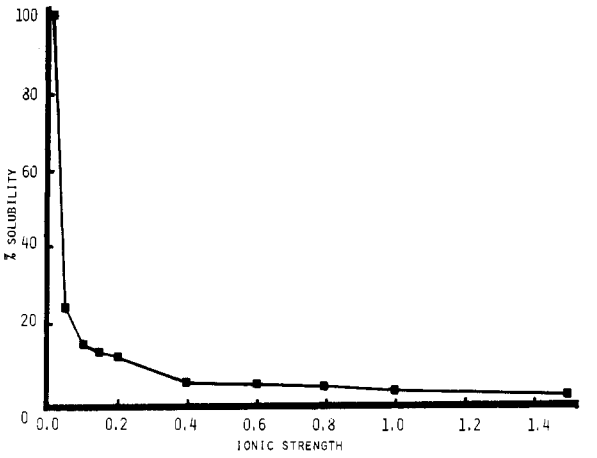

Figure 4. Solubility of basic subunits against ionic strength of NaCl at pH 5.5.

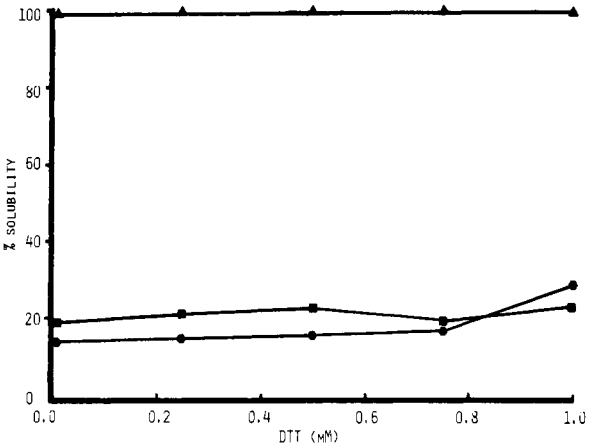

Figure 5. Solubility of basic subunits vs. the concentration of DTT at pH 4.5 (▲), 5.5 (■), and 6.0 (●).

subunits aggregated over a broad pH range between 5 and 9. These data explain the selective aggregation of basic subunits at pH 8.0. Thus, once dissociated from the acidic subunits in pH 6.5–8.0 these subunits aggregate.

Solubility of Basic Subunits. Solubility of the basic subunits was examined more extensively by using a multivariate experimental design to investigate the forces responsible for the association of the proteins. Ionic strength, pH, and reductant were varied according to a central composite design, and soluble protein was regressed against these variables by using response surface methodology.

The fitted model revealed a significant negative interaction between pH and ionic strength. The protein solubility at pHs far from the isoelectric point was reduced by the neutralization of charged groups upon the addition of salt. The solubility of basic subunits at various ionic strength of NaCl at pH 5.5 is presented in Figure 4. The basic subunits are largely salted out above an ionic strength of 0.1 M. Furthermore, the response surface model showed that the presence of reductant did not increase the aggregation of the basic subunits and in fact increased solubility slightly at pH 5.5. The solubility of basic subunits at pH 4.5, 5.5, and 6.0 as a function of dithiothreitol concentration, a strong sulfhydryl reductant, is shown in Figure 5. These data confirm that the effect of reductant and low ionic strength on thermal aggregation of 11 S is because they facilitate thermal dissociation of the quaternary structure. Subsequent aggregation of the basic subunits is spontaneous and is not a consequence of disulfide rearrangement or electrostatic attractions. This also implies that hydrophobic forces may be involved in

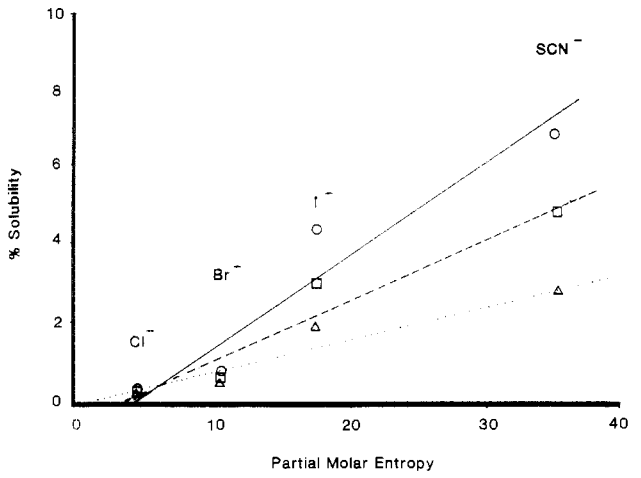

Figure 6. Solubility of basic subunits vs. the partial molar entropy of the anions chloride, bromide, iodide and thiocyanate and Na salts at 0.4 (△), 0.6 (□), and 0.8 M (○) ionic strength at pH 5.5. Solubility was measured as absorbance at 280 nm in the supernatant after centrifuging at 20000*g* for 15 min.

the aggregation of basic subunits at neutral pH.

Hydrophobic interactions are a direct consequence of a decrease in the entropy of the aqueous solvent immediately surrounding exposed alkyl groups (Frank and Evans, 1945). Nonpolar groups such as apolar amino acids in proteins tend therefore to associate in water to minimize this energetically unfavorable "structuring" of surrounding water. The nature and magnitude of hydrophobic interactions are then a direct consequence of the state of the surrounding water. The native structure of water has been shown to be progressively disrupted by the addition of anionic salts in the chaotropic series (Dandliker and de Saussure, 1971). This would predict that effects that are a consequence of the structure of water, i.e., hydrocarbon solubility and hydrophobic interactions, would be decreased also in the order of the chaotropic series. The solubility of benzene in water increases linearly with the partial molal entropies of hydration of the anions in the chaotropic series (McDevit and Long, 1952). Protein–protein interactions of a hydrophobic nature have also been shown to follow the Hofmeister series of anions (von Hippel and Schleich, 1969). Hence, the contribution of the hydrophobic effect in causing the association of proteins can be estimated by altering the structure of the water itself using the chaotropic salts. The solubility of basic subunits at pH 5.5 at ionic strength 0.4, 0.6, and 0.8 M for four representative anions in the Hofmeister series is presented in Figure 6. The very strong correlation between solubility and disruption of water structure indicates that hydrophobic forces strongly contribute to the association of basic subunits.

These data verify that basic subunits tend to readily aggregate when released from their association with the acidic subunits in the oligomeric 11 S. However, in the presence of the 7S fraction, under the conditions used, no precipitation occurs. Two possible mechanisms appear likely to account for the effect of 7 S in inhibiting this aggregation. The 7 S could interact directly with the 11S molecule and increase the stability of 11 S, thereby inhibiting the release of the basic subunits. Alternatively, the 7S molecule or its constituent monomers could interact with the basic subunits once released and by forming a complex solubilize them.

Calorimetry of 11 S and 7 S. An inevitable consequence of the first mechanism would be a change in the thermal stability of the oligomer and therefore a resolvable

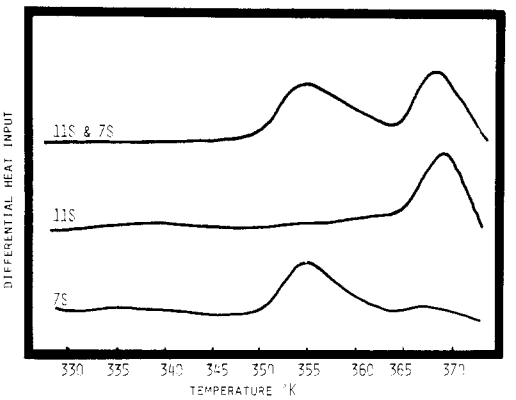

Figure 7. Differential scanning calorimetry thermograms of protein solutions (8%) of soy 11 S and 7 S in an equal-weight mixture, purified 11 S, and purified 7 S, in Tris buffer, pH 8.0, with 10 mM 2-mercaptoethanol, scanned at 20 K/min.

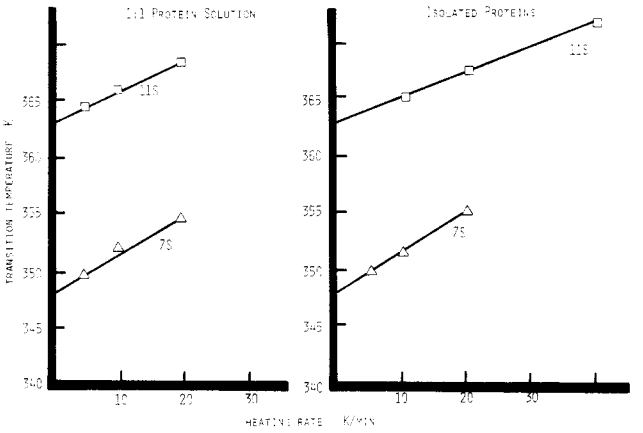

Figure 8. Transition temperatures calculated as the midpoint on the transition peak of the thermogram as a function of the scanning rate for 8% protein solutions. Transition temperature at 0 K/min scanning rate was extrapolated by linear regression.

alteration in the thermal transition of the 11S protein when examined by differential calorimetry. Thermograms of the isolated 11S subunits showed no endothermic transition below 100 °C (not shown), indicating that transitions exhibited by 11 S below this temperature must be due entirely to the dissociation of the quaternary structure.

The 7S and 11S proteins were analyzed separately and in equal mixture combinations with differential scanning calorimetry. Typical thermal profiles of these proteins are shown in Figure 7. The actual transition temperature is calculated to be the maximum on the enthalpy curve extrapolated to the zero scanning rate. The transition data for these proteins are shown in Figure 8. By linear regression, the transition temperature of both 7 S and most significantly 11 S is independent of the presence of the other. This suggests rather strongly that 7 S does not stabilize the oligomeric structure of 11 S. These data indicate that upon heating 11 S the basic subunits are liberated but fail to aggregate because the 7S and basic subunits form a soluble complex.

Reversible Cross-Linking of Heated Soy Proteins. The possible formation of a soluble complex between 7S monomers and basic subunits was explored by using a reversible cross-linking experiment to covalently link and resolve by electrophoresis the proteins that associate following heating. The 11S and 7S proteins were heated and cooled, free sulfhydryls were modified, and proteins were cross-linked by using the cleavable cross-linker dithiobis(propionimidate). The resulting proteins were electrophoresed on a slab gel under denaturing conditions

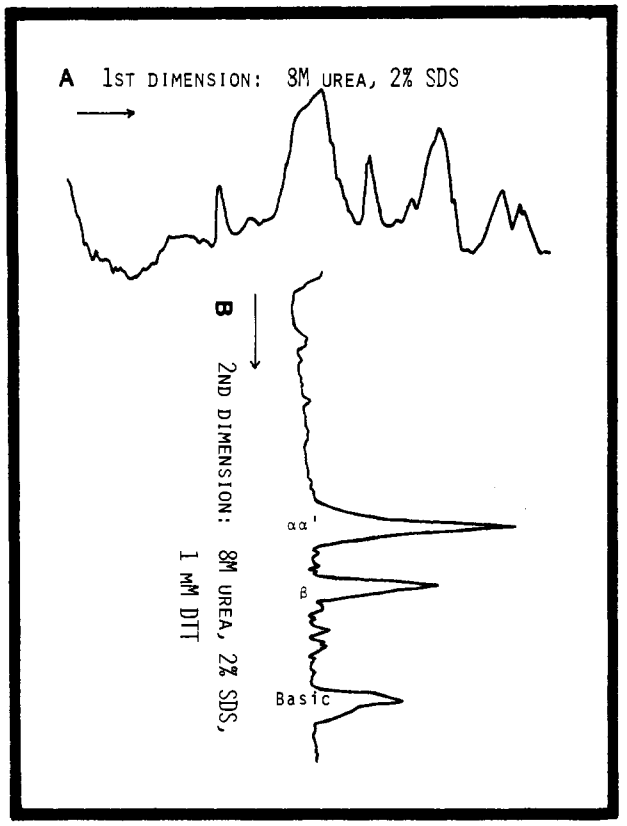

Figure 9. Densitometer scans of $NaDodSO_4$ (SDS in the figure)–polyacrylamide electrophoretic gels under dissociating conditions. (A) Heated 11 S and 7 S cross-linked by using dithiobis(propionimidate) run in the first dimension. (B) Densitometer scan of the channel corresponding to the M_r 60000 band after channel A was cut out and the bands were reductively cleaved by using 1 mM DTT, placed on a second gel, and rerun into a second dimension (see Materials and Methods).

without reductant in the first dimension. A channel containing separated proteins was then cut out of the first gel, incubated in denaturing buffer with reductant to split the cross-linking agent, and rerun in a second $NaDodSO_4$ gel. The results are shown in Figure 9. A large band of poorly resolved protein is evident at an R_f that corresponds to a protein of M_r of 60000. When this band was reduced and separated in the second dimension, it was found to contain three bands. These correpond electrophoretically to the α and β monomers of 7 S and the basic subunit of 11 S. This profile could have resulted from linkage of the M_r 42000 β monomer and the M_r 19000 basic subunit into a complex of M_r 60000 that would comigrate with the α monomers. We have not as yet been able to distinguish cross-linking products of the α monomers with basic subunits. This could indicate either insufficient numbers of such complexes or the absence of appropriately located amine groups on the α monomers available for reaction with the cross-linking agent.

CONCLUSIONS

While stability of the 11S protein is undoubtedly a consequence of extensive intermolecular and intramolecular disulfide and noncovalent associations in the subunits, release of the subunits themselves in a complex protein mixture does not lead inevitably to precipitation of the peptides. The results presented here indicate that interactions between monomers released from the native oligomeric structure of 11 S lead to novel and in this case soluble structures. This has obvious implications with regard to the functional properties of heated soy proteins.

The heat stability of soy protein is an obstacle to its universal utilization as a functional food protein. Portions of the soy protein that because of their electrostatic and hydrophobic components would suit them ideally for thermal aggregation, namely the 11S basic subunits, are prevented from doing so by an interaction and complex formation with one or all of the 7S particles present. Manipulation of this interaction and improvement of the thermal properties of soy require an understanding of the nature of the mechanism of this interaction, which is currently being studied.

LITERATURE CITED

Badley, R. A.; Atkinson, D.; Hauser, H.; Oldani, G.; Green, J. P.; Stubbs, J. M. *Biochim. Biophys. Acta* **1975**, *412*, 214.
Catsimpoolas, N. *FEBS Lett.* **1969**, *4*, 259.
Catsimpoolas, N.; Funk, S. F.; Meyer, E. W. *Cereal Chem.* **1970**, *47*, 221.
Catsimpoolas, N.; Kenney, J. A.; Meyer, E. W.; Szuhaj, B. F. *J. Sci. Food Agric.* **1971**, *47*, 311.
Cochran, W. G.; Cox, G. M. "Experimental Designs", 2nd ed.; Wiley: New York, 1957; p 335.
Dandliker, W. B.; de Saussure, V. A. In "The Chemistry of Biosurfaces"; Hair, M. L., Ed.; Marcel Dekker: New York, 1971; Vol. 1, p 1.
Frank, H. S.; Evans, M. W. *J. Chem. Phys.* **1945**, *13*, 507.
Hashizume, K.; Watanabe, T. *Agric. Biol. Chem.* **1979**, *43*, 683.
Hermans, J. J.; Scheraga, H. A. *J. Am. Chem. Soc.* **1961**, *83*, 3283.
Hermansson, A. M. In "Functionality and Protein Structure"; Pour-El, A., Ed.; American Chemical Society: Washington, DC, 1979; ACS Symp. Ser. No. 92, p 81.
Kamata, Y.; Okubo, K.; Shibasaki, K. *Agric. Biol. Chem.* **1979**, *43* (6), 1219.
Kinsella, J. E. *J. Am. Oil Chem. Soc.* **1979**, *56*, 242.
Kitamura, K.; Takagaki, T.; Shibasaki, K. *Agric. Biol. Chem.* **1976**, *39*, 945.
Laemmli, U. K. *Nature (London)* **1970**, *227*, 680.
Lewis, B.; Chen, J. In "Functionality Protein Structure"; Pour-El, A., Ed.; American Chemical Society: Washington, DC, 1979; ACS Symp. Ser. No. 92, p 27.
Matthews, C. R.; Westmoreland, D. G. *Biochemistry* **1975**, *14*, 4532.
McDevit, W. F.; Long, F. A. *J. Am. Chem. Soc.* **1952**, *74*, 1773.
Peters, K.; Richards, F. M. *Annu. Rev. Biochem.* **1977**, *46*, 523.
Privalov, P. L. *Adv. Protein Chem.* **1979**, *33*, 167.
Thanh, V. H.; Shibasaki, K. *J. Agric. Food Chem.* **1976**, *24*, 1117.
Thanh, V. H.; Shibasaki, K. *Biochim. Biophys. Acta* **1977**, *490*, 370.
von Hippel, P. H.; Schleich, T. In "Structure and Stability of Biological Macromolecules"; Timasheff, S. N.; Fasner, G. D., Eds.; Marcel Dekker: New York, 1969; Vol. 2, p 417.
Wolf, W. J.; Tamura, T. *Cereal Chem.* **1969**, *46*, 331.

Received for review November 6, 1981. Revised manuscript received May 20, 1982. Accepted June 11, 1982.