Studies on factors affecting flavor binding to soy proteins will be described in the following paper (Damodaran and Kinsella, 1981).

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Interaction of Carbonyls with Soy Protein: Conformational Effects

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The relationship between the structure and ligand binding by soy protein was studied by using an equilibrium dialysis method. In isolated systems, the 11S and 7S protein fractions of soy exhibited different binding affinities for 2-nonanone. While the binding affinity of the 7S fraction was the same as that of whole soy protein, the 11S fraction exhibited almost no affinity for 2-nonanone. These differences are interpreted in terms of the structural differences in these two proteins. Urea and chemical modification (succinylation) profoundly affected both the binding affinity and the binding capacity of soy protein for ketones. The decrease in the binding constant in the presence of urea and succinylation was due to structural changes in the protein as evidenced by the changes in the fluorescence behavior of soy protein.

In a preceding paper (Damodaran and Kinsella, 1981a) we demonstrated that the interaction of carbonyls with soy protein is hydrophobic in nature. The binding affinity depended on the chain length as well as the position of the keto group in the chain. Comparison of the binding constants of 2-nonanone interaction with bovine serum albumin (Damodaran and Kinsella, 1980a) and soy protein revealed that the interaction of a hydrophobic ligand with a protein depends on the structural state of the binding sites. Because the molecular structures of different proteins are very dissimilar, the hydrophobicity of the binding sites and hence the magnitude of the hydrophobic interactions with the ligand also vary. This indirectly implies that by altering the structural state of the binding sites in soy protein, it may be possible either to increase or to decrease the binding affinity of carbonyls for soy protein. In fact, the increase in the binding constant for 2-nonanone with heat-denatured soy protein is a simple manifestation of structural differences between heat-treated and native soy protein. Since the magnitude of hydrophobic interactions is greater at higher temperatures, the heat treatment would increase the intramolecular hydrophobic interactions in the protein and thus create stronger hydrophobic regions. Because the ultimate objective of this research is to determine procedures to eliminate the bound off-flavors from soy protein, an approach based on the reversible destabilization of the hydrophobic regions in soy protein is desired.

The major driving force for hydrophobic interactions in general is the structural state of the liquid water in the presence of hydrocarbons (Kauzmann, 1959). It is the thermodynamically unfavorable lower entropy state of the liquid water in the presence of apolar groups which is responsible for both the formation of hydrophobic regions in proteins and the association of hydrophobic ligands with proteins (Kauzmann, 1959; Tanford, 1973; Lewin, 1974). Logically this implies that one can alter the tertiary and quaternary structures in proteins by altering the structural state of the solvent. Previously we have shown that the binding of 2-nonanone to bovine serum albumin was affected by chaotropic salts (Damodaran and Kinsella, 1980b, 1981b). This was attributed to destabilization of the hydrophobic binding sites by chaotropic anions via changes in the water structure. Logically one can expect similar trends with structural perturbants like urea and guanidine hydrochloride. The present paper deals with an attempt to understand the relationship between the structural states of soy protein and its interaction with carbonyls. 2-Nonanone was selected as representative of carbonyls for this purpose.

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EXPERIMENTAL SECTION

Materials. Whole soy protein and soy 11S and 7S protein fractions were isolated from defatted, low heat treated soy flour (Central Soya, Chicago, IL, lot no. 878, code 3040). Spectral-grade isooctane was obtained from Fisher Scientific Co. (Fair Lawn, NJ). 2-Nonanone (99+%) was obtained from Aldrich Chemicals (Milwaukee, WI). All other chemicals used in this study were reagent grade.

Isolation of Soy Proteins. Whole soy protein was isolated from soy flour by isoelectric precipitation as described in the preceding paper (Damodaran and Kinsella, 1981a). The soy 11S and 7S protein fractions were isolated from soy flour by using the method of Thanh and Shibasaki (1976). The soy 11S fraction was further purified by Sepharose 6B chromatography. Sepharose 6B (Sigma Co., MO) was packed in a 2.5 cm diameter glass column to a bed volume of 700 mL. The column was equilibrated with the buffer (35 mM phosphate buffer, pH 7.6, containing 10 mM 2-mercaptoethanol, 0.4 M NaCl, and 0.02% sodium azide). After the protein solution (0.5 g/10 mL) was loaded, the column was washed with the above buffer at room temperature. The flow rate was 35 mL/h. Ten-milliliter fractions were collected, and the tubes containing 11S protein were pooled together, dialyzed against water at pH 8.0 for 48 h at 4 °C, and then lyophilized. In the case of the 7S protein fraction, no further purification was done and it was used as such for binding studies. The purity of this sample, as determined from $NaDodSO_4$–polyacrylamide gel electrophoresis, was about 90%. The contaminants were 11S and 2S protein fractions.

Protein Solutions. For binding studies the protein solutions were made in 30 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 0.02% sodium azide. Protein at 1% concentration was used in all the experiments. An absorptivity of 8.02 at 280 nm for a 1% solution was used to estimate the protein concentration (Thanh and Shibasaki, 1976).

Binding Measurements. The interaction of 2-nonanone with soy proteins was studied by the equilibrium dialysis method as described in the preceding paper (Damodaran and Kinsella, 1981a). The binding data were analyzed by using the double-reciprocal equation

$$1/\bar{\nu} = 1/n + 1/(nK[\mathrm{L}])$$

where $\bar{\nu}$ is the molal ratio of binding, n is the total number of binding sites, [L] is the free ligand concentration in molar units, and K is the intrinsic binding constant.

Succinylation. Succinylation of soy proteins with succinic anhydride was carried out as follows (Franzen and Kinsella, 1976). To a solution of soy protein (1%), succinic anhydride was added (at 2:1 weight ratio of succinic acid to protein) with stirring over a period of 1 h while the pH was maintained between 8.0 and 8.5 by addition of 2 N NaOH. After the pH was stabilized to about pH 8.0–8.5, the solution was stirred for another 20 min. Under these conditions all the lysyl groups in soy protein were succinylated (Shetty and Kinsella, 1979). The solution was then adjusted to pH 4.2. The precipitated protein was separated by centrifuging at 10000g for 10 min, washed twice with water, and then dissolved in water at pH 8.0. The solution was dialyzed against water at pH 8.0 for 24 h and lyophilized.

Fluorescence Spectra. The fluorescence spectra of proteins were measured by using a Perkin-Elmer Model 650-40 fluorescence spectrophotometer equipped with a recorder. Protein solutions were excited at 285 nm, and the fluorescence spectra were scanned from 300 to 400 nm.

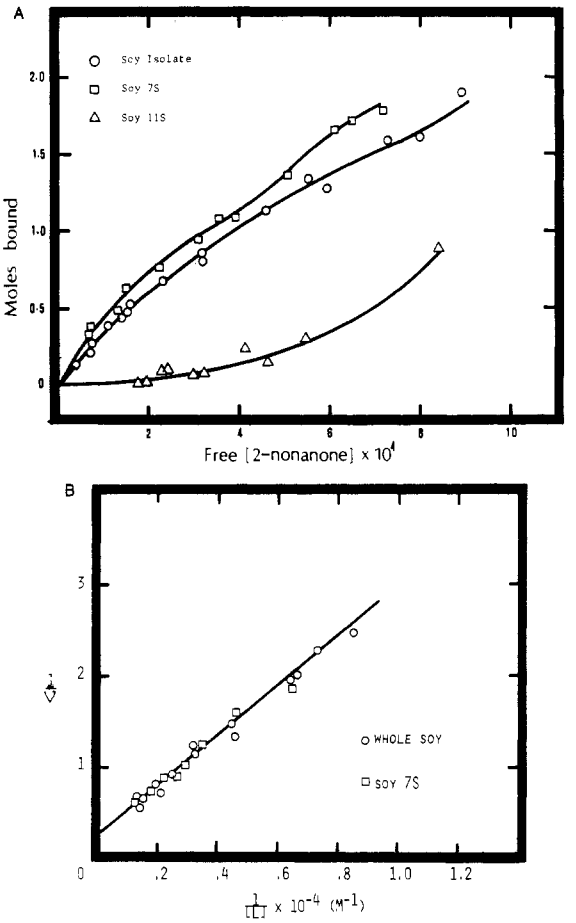

Figure 1. (A) Binding of 2-nonanone to whole soy protein isolate (○), soy 7S fraction (□), and soy 11S fraction (△) in 0.03 M Tris-HCl buffer, pH 8.0, at 25 °C. (B) Double-reciprocal plot of the binding of 2-nonanone to whole soy protein (○) and soy 7S protein fraction (□). $\bar{\nu}$ is the number of moles of ligand bound per mole of protein, and [L] is the free ligand concentration in moles per liter.

RESULTS AND DISCUSSION

Binding of 2-Nonanone to Soy 11S and 7S Protein Fractions. Soy protein isolate consists of two major protein fractions, 11 S and 7 S, based on their sedimentation coefficients. The molecular structure as well as the functional behavior of these two proteins is quite different (Smith and Circle, 1978). To understand whether these two fractions differ in their interaction with carbonyls, we studied the binding of 2-nonanone to these two major protein fractions.

The binding isotherms for 2-nonanone with whole soy protein and soy 11S and 7S fractions are shown in Figure 1A. The binding affinity of 2-nonanone for whole soy protein and soy 7S protein fraction were almost identical. In fact, the double-reciprocal plot of the data (Figure 1B) exhibited identical slopes and intercepts, suggesting that the binding constant and the total number of binding sites were the same. On the other hand, soy 11S protein fraction exhibited very weak binding affinity for 2-nonanone (Figure 1A). The double-reciprocal plot for 2-nonanone binding to the 11S protein is not presented here since the slope of such a plot was almost parallel to the ordinate, suggesting that the binding affinity was almost zero.

Although the binding studies with 7 S and 11 S were performed with isolated samples, it may be speculated that in the whole soy protein, in which the 11S and 7S proteins exist in a definite molar ratio, the carbonyls may preferentially interact with the 7S fraction. If one assumes that

the free energy of interaction of 2-nonanone with whole soy protein is the summation of free energies of interaction with 7 S and 11 S, i.e.

$$\Delta G_{soy} = \Delta G_{7S} + \Delta G_{11S}$$

then, since the interaction with 11 S is almost zero, one would get the same free energy change for both 7S protein and whole soy protein. This is clearly reflected in the binding isotherms (Figure 1A). In other words, the major protein fraction responsible for off-flavor binding in soy may be the 7S protein fraction. This preferential interaction may be due to differences in the molecular structures of 7S and 11S proteins. The 7S protein consists of three subunits and has a molecular weight of about 175 000 (Thanh and Shibasaki, 1978), whereas the 11S protein has a molecular weight of 320 000 and contains six acidic and six basic subunits (Kitamura et al., 1976; Badley et al., 1975). The spatial arrangement of the subunits in these two proteins may be such that the 7S protein may have hydrophobic regions which are accessible for ligand binding, whereas in the case of 11S protein such hydrophobic regions may be buried inside the protein and hence may neither be available nor accessible for interaction with the ligand. The 11 S contains about 21 disulfide linkages (Badley et al., 1975) compared to none in the 7 S (Thanh and Shibasaki, 1978). In fact, such high content of disulfide linkages in 11 S may be responsible for its high resistance to heat denaturation compared to 7 S. It may also be possible that the network of disulfide linkages may render the hydrophobic regions inaccessible for interacting with the ligand.

If the weak interaction between 11 S and 2-nonanone was due to its unique quaternary structure, then changes in this structure should result in either an increase or a decrease in the binding affinity for 2-nonanone. It is known that the oligomeric structure of soy 11 S is dependent on ionic strength of the medium. While at 0.5 M ionic strength the protein has a sedimentation coefficient of 11 S, below 0.1 M ionic strength it dissociates into "half-11 S" (Koshiyama, 1972). In fact, such dissociation is clearly observed in polyacrylamide gel electrophoresis patterns where about 0.05 M Tris–glycine buffer is used. Since the binding isotherms presented in Figure 1A were obtained in 30 mM Tris-HCl buffer, pH 8.0, it may be assumed that the 11 S was in the half-11 S form. The interaction of 2-nonanone with soy 11 S at 0.5 M ionic strength is shown in Figure 2A. The same data are presented in the form of double-reciprocal plots in Figure 2B. There is a dramatic increase in the binding affinity at 0.5 M compared to 0.03 M ionic strength. This effect seems to be independent of the type of the ion and dependent only on the ionic strength. The intercept of the double-reciprocal plot (Figure 2B) suggests that there are about eight binding sites in soy 11 S at 0.5 M ionic strength and that it has an intrinsic binding constant of 290 M^{-1} for 2-nonanone. This clearly suggests that the increase in the binding constant is due to ionic strength induced association of 11 S. At 0.5 M ionic strength, 11 S exists as two hexagons, placed on one another in the form of a hollow cylinder (Badley et al., 1975). It is possible that the cavity at the center of this hexagonal cylinder may act as the binding site for 2-nonanone. Whatever the reason for the absence of interaction between 2-nonanone and 11 S at 0.03 M ionic strength, the results imply that if one isolates the 11S protein from soy, it should be devoid of off-flavors originally present in the whole soy protein. Since the 11 S is about 35% of the total proteins in soy (Thanh and Shibasaki, 1976), the commercial preparation

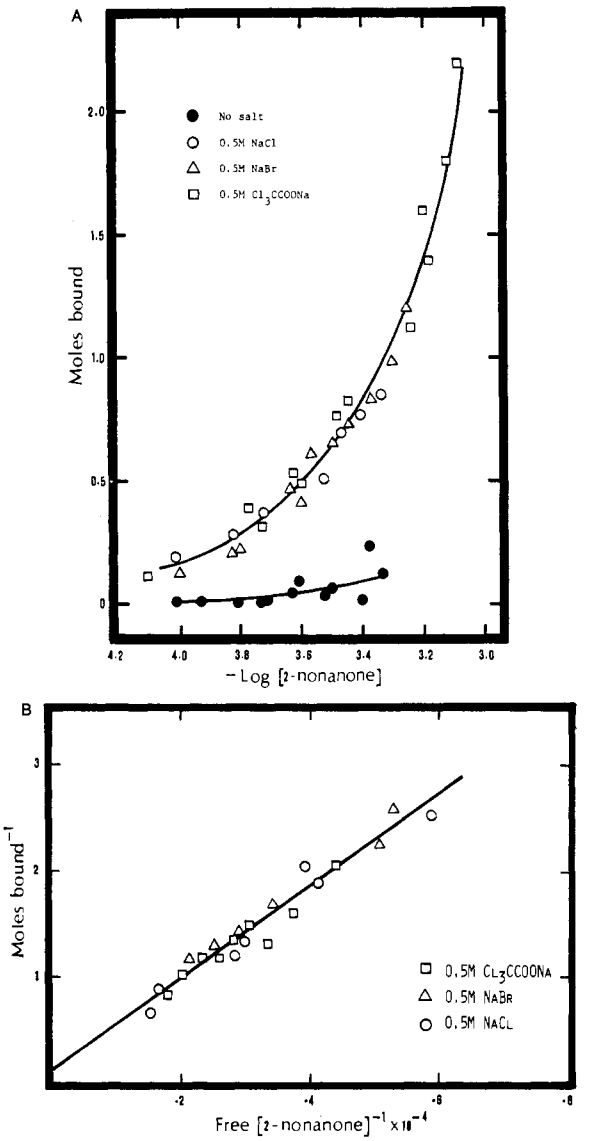

Figure 2. (A) Effect of ionic strength on the binding of 2-nonanone to soy 11S protein in 0.03 M Tris-HCl buffer, pH 8.0. Experiments were done at 0 and 0.5 M concentrations of various salts. (●) No salt; (○) 0.5 M NaCl; (△) 0.5 M NaBr; (□) $Cl_3CCOONa$. (B) Double-reciprocal plots of the binding of 2-nonanone to soy protein at 0.5 M concentrations of NaCl (○), NaBr (△), and $Cl_3CCOONa$ (□).

of the off-flavor free 11S protein may be worthwhile for several functional applications.

Effect of Urea. It is obvious from the above results that the hydrophobic interaction between a ligand and a protein is dependent on the steric compatability as well as the hydrophobicity of the binding sites. Hence, it follows that any change in the structural state of the binding sites should profoundly affect the hydrophobic interaction between the ligand and the protein. Such structural changes can be induced by urea.

The effect of urea on the binding of 2-nonanone to soy protein is shown in Figure 3. The slopes of the double-reciprocal plots increase with urea concentration, suggesting that the binding affinity of soy protein for 2-nonanone decreases with an increase in urea concentration. Such changes in the binding affinity may be due to changes in the protein structure in the presence of urea. This is also evident from the observation that the double-reciprocal plots of the binding data at various concentrations of urea do not exhibit a clear intercept. This, apart from leading to an uncertainty in the estimation of the number

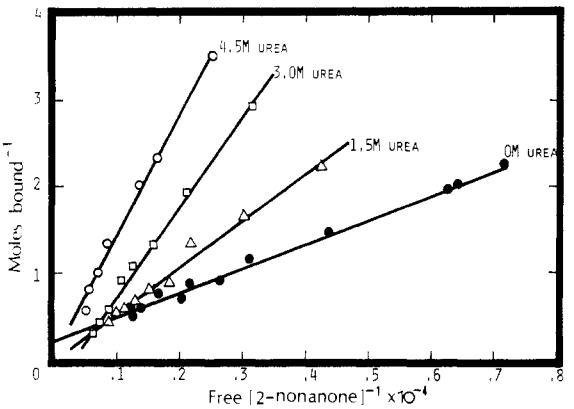

Figure 3. Effect of urea on the binding of 2-nonanone to soy protein at 25 °C in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol.

of binding sites, implies that as the urea concentration is increased, the unfolding of the protein creates almost an infinite number of binding sites having very low binding affinities. To understand the relationship between urea-induced structural changes in the protein and the changes in the binding affinity for 2-nonanone, we studied the fluorescence behavior of soy protein.

The fluorescence spectra of soy protein at various concentrations of urea are shown in Figure 4A. The native soy protein exhibits maximum fluorescence intensity at 337 nm, suggesting that the fluorescence is mainly due to tryptophan residues. The fluorescence intensity decreases with urea concentration up to 4 M and then again increases above 4 M. On the other hand, the λ_{max} of fluorescence emission undergoes a red shift up to about 6 M urea and apparently remains the same above 6 M. The red shift in the λ_{max} of fluorescence emission clearly demonstrates that there is unfolding of the protein which exposes the tryptophan residues from the interior of the protein to the aqueous environment. Such exposure of tryptophan residues to the polar solvent environment would shift the λ_{max} of fluorescence to higher wavelength (Stryer, 1968). Since in most proteins tryptophan residues are found in hydrophobic regions, the above result also suggests that urea destabilizes the hydrophobic regions in soy protein. It is known that the denaturing action of urea involves a hydrophobic mechanism that favors exposure of nonpolar residues in the protein to solvent environment (Wetlaufer et al., 1964). This is basically due to changes in the water structure in the presence of urea, which thermodynamically favors the transfer of apolar groups to the aqueous environment and thus destabilizes the hydrophobic regions in the protein (Damodaran and Kinsella, 1981b).

The relationship between the binding affinity and the shift in λ_{max} of fluorescence emission as a function of urea concentration is shown in Figure 4B. Because of the uncertainty in the determination of number of binding sites, the binding constant is presented here as nK where n is the number of binding sites and K is the intrinsic binding constant. Despite the increase in the n value, the value of nK decreases with urea concentration, whereas the λ_{max} of fluorescence undergoes a red shift with urea concentration. There is a striking correlation between the shift in the λ_{max} and the decrease in the binding constant, suggesting that the changes in the structural state of the protein has a profound effect on the interaction of carbonyls with soy protein.

Above 4 M urea concentration the rate of decrease in the binding constant as well as the shift in the λ_{max} is minimal. This suggests that the major structural changes

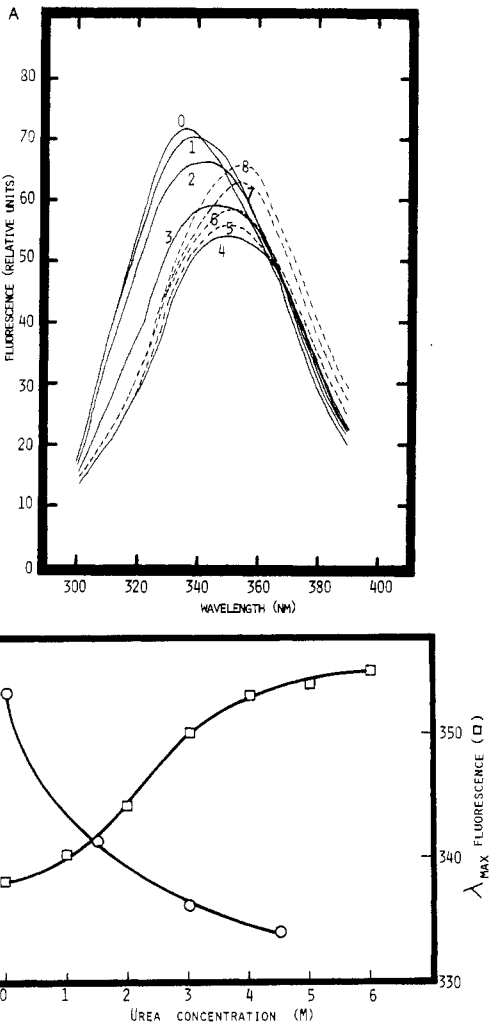

Figure 4. (A) Fluorescence spectra of soy protein at various concentrations of urea in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. The numbers on the curves indicate the urea concentration. The wavelength maxima of fluorescence at various urea concentrations were 337 nm (control), 340 nm (1 M), 344 nm (2 M), 350 nm (3 M), 353 nm (4 M), 354 nm (5 M), 356 nm (6 M), and 357 nm (7 and 8 M). (B) Relationship between the shift in the wavelength maximum of fluorescence emission and the binding affinity of soy protein for 2-nonanone at various urea concentrations. n is the number of binding sites, and K is the intrinsic binding constant.

that affect the binding constant occur below 4 M urea. The major structural changes that occur in urea up to 4 M involve only dissociation of the subunits while the unfolding of the subunits occur only above 5 M (Lillford, 1978). This supports our previous suggestion that the existence of the binding sites in soy proteins is due to unique orientations of the subunits in the quaternary structures and not the tertiary structures of individual subunits. In other words, the association of subunits may create hydrophobic cavities at the subunit interfaces which act as binding sites for carbonyls. This is consistent with the effect of ionic strength on 2-nonanone binding by 11 S. In practical terms this means that if the subunits of soy proteins are reversibly dissociated by some means, it should be possible to remove the carbonyls originally bound to the protein. The advantage of such an approach is that upon removal of the reagent the protein may be reversed to the native state and thus retain its functional properties. This can be achieved by treating soy protein or soy flour with 1–2 M urea, followed by dialysis to remove urea as well as the previously bound carbonyls.

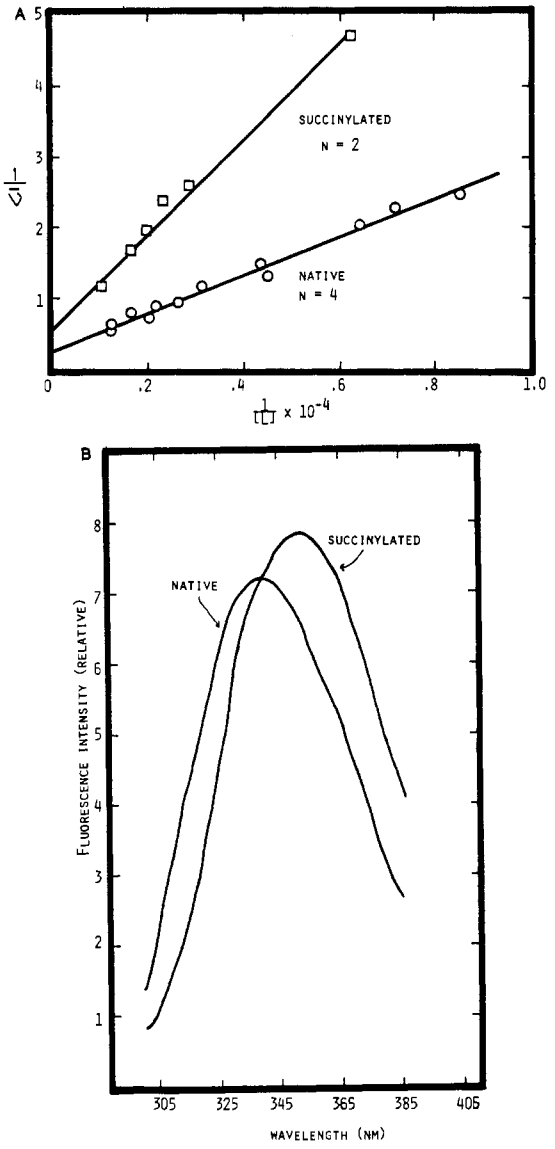

Figure 5. (A) Effect of succinylation of soy protein on the binding of 2-nonanone at 25 °C, in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. (B) Fluorescence spectra of native and succinylated soy protein in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. The λ_{max} of fluorescence of native soy is 337 nm and that of succinylated soy is 353 nm.

Effect of Succinylation. The effect of succinylation of soy protein on the binding of 2-nonanone is shown in Figure 5A. The intercept suggests that there are about two binding sites for 2-nonanone in succinylated soy compared to four binding sites in the native protein. But the intrinsic binding constants for the native and succinylated soy proteins are almost the same. Succinylated soy protein has a binding constant of 850 M^{-1} for 2-nonanone compared to 930 M^{-1} for native soy protein. This suggests that the conformational changes in soy protein upon succinylation destroy half of the binding sites originally present. Such conformational changes are clearly reflected in the fluorescence spectrum of succinylated soy protein (Figure 5B). The λ_{max} of fluorescence is shifted from 337 to 353 nm, suggesting that the tryptophan residues are more exposed to the solvent, which may be indicative of destabilization of the hydrophobic regions in the protein. Since the conformational change by succinylation is due to electrostatic repulsion, one would expect a complete dissociation and denaturation of the native protein upon succinylation. In fact, it has been shown with arachin (Shetty and Rao, 1978) and soy 11 S (Rao and Rao, 1979) that above 80% succinylation these proteins completely dissociated into individual subunits and possibly assumed a more random coil structure. But the binding studies with 2-nonanone suggest that only half of the total number of binding sites in soy protein are destroyed by succinylation. This implies that some of the protein fractions in soy (possibly the 7S fraction) undergo only partial destabilization upon succinylation and still retain part of its ligand binding capacity.

The above results suggest that there is a good correlation between the structure of soy protein and its ability to bind carbonyl compounds. By reversibly altering the quaternary structures, it may be possible to remove the bound off-flavors from this protein. Studies to test this are in progress.

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