# Interaction of Carbonyls with Soy Protein: Thermodynamic Effects

Srinivasan Damodaran* and John E. Kinsella

The interaction of carbonyls with soy protein was investigated by using an equilibrium dialysis method. At saturation level, soy protein exhibited four binding sites for all the carbonyls studied. The binding constant increased with the chain length of the ligand by 3 orders of magnitude for each methylene group increase in the chain. The favorable change in the hydrophobic free energy was 550 cal/mol of $CH_2$ residue. The position of the carbonyl group decreased the hydrophobic free energy by 105 cal/mol for each shift from the terminal 1 position to the middle of the chain. While the binding affinity was independent of temperature above 25 °C, a drastic increase in the binding affinity was observed at 5 °C. Partial denaturation of soy protein increased the binding constant. Thermodynamic analysis of the binding of carbonyls with soy protein revealed that the interaction is relatively weak.

One of the major problems limiting the utilitzation of oilseed proteins, e.g., soy and peanut proteins, in conventional foods is the off-flavors associated with these preparations (Kalbrener et al., 1971; Kinsella, 1978; Kinsella and Damodaran, 1980). The off-flavors are mainly due to aldehydes, ketones, and alcohols generated by the action of lipoxygenase on unsaturated fatty acids (Kalbrener et al., 1974; Wolf, 1975). These carbonyls interact with the storage proteins in the seed and resist their removal by conventional methods (Arai et al., 1970).

Information concerning the interactions of flavor compounds with proteins is limited. A few qualitative studies concerning the interaction of flavors with proteins in aqueous systems have been done. Nawar (1971) showed that the presence of gelatin decreased the volatility of methyl ketones in aqueous systems. By headspace analysis, Gremli (1974) showed that in aqueous dispersion of soy protein and aldehydes, the volatility of aldehydes decreased compared to that with the absence of soy protein. The decrease in the volatility increased with the chain length of the aldehyde, indicating that the magnitude of the interaction between the protein and aldehyde depended on the chain length. Franzen and Kinsella (1974) found that the addition of proteins to model systems containing water and flavor consistently caused a decrease in the headspace concentration.

While the above studies described the qualitative effects of proteins on the volatility of flavor compounds, they did not deal with the quantitative aspects of flavor–protein interaction. there are some conflicting reports in the literature regarding the nature and magnitude of flavor–protein interactions (Arai et al., 1970; Beyeler and Solms, 1974). These may be attributed to inadequate methods and inappropriate systems used. There is a need for a systematic study to understand the fundamental nature of these interactions. such information is needed to devise appropriate methods for the removal of off-flavors from plant proteins and to facilitate flavoring of fabricated high protein foods (Kinsella, 1979). When the objective is to solve the off-flavor problems in oilseed proteins, then the molecular nature of the interaction of carbonyls with proteins in their native state must be understood. Studies with denatured proteins may not be indicative of the true molecular nature of interaction of carbonyls because the carbonyls produced from lipoxygenase activity during the processing of the beam must interact with proteins in their native state. The present paper deals with the thermodynamics of interaction of carbonyls with soy protein.

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## EXPERIMENTAL SECTION

**Materials.** Whole soy protein was prepared from defatted and low heat treated soy flour (Central Soya, Chicago, IL; lot no. 878, code 3040). Spectral-grade isooctane was purchased from Fisher Scientific Co. (Fairlawn, NJ). 2-Nonanone (99+%), 2-octanone (98%), nonanal (98%), and 5-nonanone were obtained from Aldrich Chemicals (Milwaukee, WI). The purity of these carbonyls was checked by gas chromatography. All other chemicals used in this study were of reagent grade. Distilled and deionized water was used in all the experiments.

**Isolation of Soy Protein.** Whole soy protein was prepared from low heat treated soy flour by isoelectric precipitation. The soy flour was extracted with 30 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. The meal to buffer ratio was 1:20. The solution was centrifuged and the supernatant was adjusted to pH 4.8 with 2 N HCl. The precipitate was redissolved in the Tris-HCl buffer and dialyzed against water at pH 8.0 for 24 h and then lyophilized.

**Protein Solutions.** For binding studies with whole soy protein, 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% concentrations 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).

**Measurement of Binding.** The interaction of ketones and aldehydes with soy protein was studied by using a equilibrium dialysis method. Acrylic cells of equal volume, separated by a membrane (Spectropor-2) and clamped together, were used. In a typical experiment, 3 mL of protein solution was placed on one side of the membrane and 3 mL of the buffer containing a known amount of the ligand on the other side. The cells were shaken at the required temperature for at least 18 h to attain equilibrium. At the end of the equilibrium period, 1 mL of the solution from each side of the membrane was drawn out and placed in vials containing 1 mL of isooctane. The ligand from the aqueous phase was extracted into the isooctane phase by shaking. Since the binding is reversible, and the aqueous/isooctane partition coefficient of carbonyls is of the order of $10^{-3}$, all of the ligand from the aqueous phase was extracted into isooctane phase. In fact, a second extraction of the aqueous phase containing protein did not contain any ligand. The concentration of the ligand in the isooctane phase was determined by gas

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Table I. Thermodynamic Constants for the Binding of Carbonyls to Soy Protein at 25 °C

| ligand | type of soy preparation | $n$ | $K_{eq}$, $M^{-1}$ | $\Delta G$, kcal/mol |
|---|---|---|---|---|
| 2-heptanone | native | 4 | 110 | −2.781 |
| 2-octanone | native | 4 | 310 | −3.395 |
| 2-nonanone | native | 4 | 930 | −4.045 |
| 2-nonanone | part. denatured | 4 | 1240 | −4.215 |
| 2-nonanone | succinylated | 2 | 850 | −3.992 |
| 5-nonanone | native | 4 | 541 | −3.725 |
| nonanal | native | 4 | 1094 | −4.141 |

chromatography. The difference in the concentration of the ligand on either side of the membrane represented the amount of the ligand bound to the protein. From the knowledge of the amount of protein and the amount of ligand bound to the protein, the molal ratio of binding was calculated as the number of moles of ligand bound per 100 000 g of soy protein. The ligand concentration of the buffer side of the membrane represented the free ligand concentration ([L]).

**Treatment of the Binding Data.** The binding data were analyzed by using the double-reciprocal equation

$$1/\nu = 1/n + 1/(nK[L])$$

where $\bar{\nu}$ is the number of moles of ligand bound per mole of the protein, [L] is the free ligand concentration, $n$ is the total number of binding sites in the protein, and K is the intrinsic binding constant.

**Gas Chromatography.** A Perkin-Elmer Model 900 gas chromatograph equipped with flame ionization detector was used. Stainless steel column ($^1/_8$ in. diameter and 10-ft length) packed with apiezon (10%) coated on Chromosorb was used. The gas flow rates were as follows: hydrogen, 15 mL/min; oyxgen, 300 mL/min; nitrogen, 40 mL/min.

## RESULTS AND DISCUSSION

The main purpose of this study was to understand the nature of binding of off-flavors to undenatured soy protein because studies with denatured protein may not be indicative of the true molecular nature of interaction of carbonyls with soy protein in the seed.

The binding isotherms for the binding of 2-heptanone, 2-octanone, and 2-nonanone to soy protein are shown in Figure 1A. The same data are presented in the form of double-reciprocal plots in Figure 1B. Within the experimental error, the intercept in Figure 1B for 2-heptanone, 2-octanone, and 2-nonanone is the same. The value of this intercept, which is the reciprocal of the total number of binding sites in the protein, suggests that there are about four to five binding sites for methyl ketones in the native soy protein (on the basis of 100 000 molecular weight). The slopes (Figure 1B), which give the reciprocal of the binding constants [$1/(nK)$], indicate that the binding affinity of ketones for soy protein increases with an increase in the chain length. The values of the equilibrium binding constants and the free energies of interaction for many carbonyls are presented in Table I. It may be noted that for each increment in the chain length, the binding constant increases 3-fold with a corresponding change in the free energy of about −600 cal/$CH_2$ residue (Table I). It may be mentioned that in the case of interaction of methyl ketones with bovine serum albumin, the binding constant increased 3-fold for each methylene group increment in the chain, with a corresponding change in the free energy of about −550 cal/$CH_2$ residue (Damodaran and Kinsella, 1980). In fact, in the case of hydrophobic interactions, it has been predicted that the binding affinity would increase by factors of 2–3 for each additional methylene group

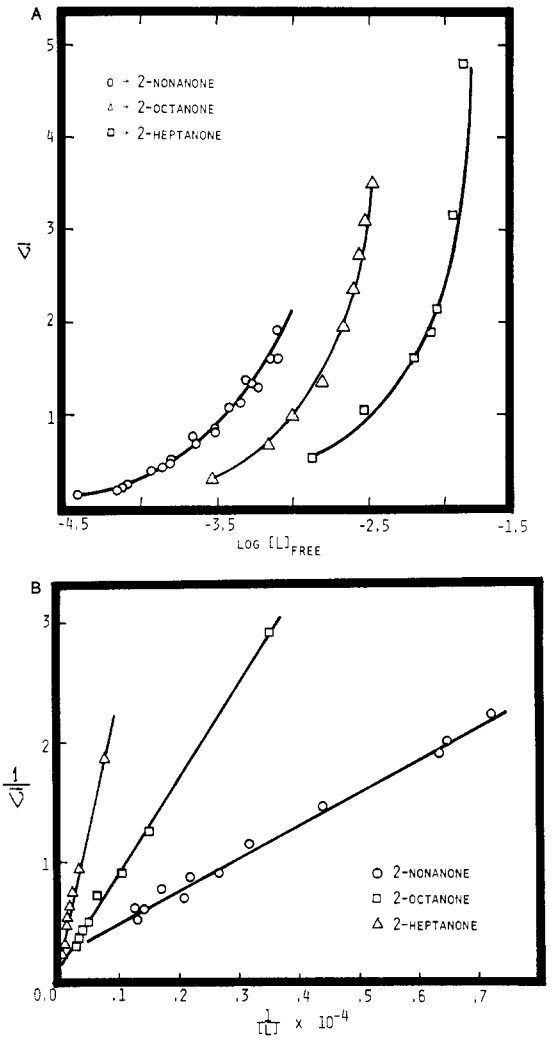


**Figure 1.** (A) Binding isotherms of 2-heptanone (□), 2-octanone (Δ), and 2-nonanone (O) to soy protein at 25 °C in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. $\bar{\nu}$ is the number of moles of ligand bound per mole of soy protein (on the basis of 100 000 molecular weight), and [L] is the free ligand concentration in moles per liter. (B) Double-reciprocal plot of the binding of 2-heptanone, 2-octanone, and 2-nonanone to soy protein at 25 °C in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. $\bar{\nu}$ is the number of moles of ligand bound per mole of protein, and [L] is the molar concentration of the free ligand.

(Wishnia, 1962, 1963). Abraham (1980) has shown that in model systems the hydrophobic free energy contribution for the transfer of a methylene group from water to an apolar solvent is about −540 cal/mol of $CH_2$. This is in agreement with our results. This evidently suggests that the binding is hydrophobic in nature. The negative change in the free energy of interaction implies that the interaction between carbonyls and soy protein is spontaneous and thermodynamically favorable.

Although the magnitude of the change in the binding constant is 3-fold for each methylene group for both bovine serum albumin and soy protein, the absolute values of the binding constants for various methyl ketones vary significantly. For example, the intrinsic binding constant for 2-nonanone to bovine serum albumin is about 1800 $M^{-1}$ (Damodaran and Kinsella, 1980), whereas with soy protein it is only 930 $M^{-1}$. This may be due to differences in the structure of these two proteins. Bovine serum albumin has six binding sites and these are highly hydrophobic. On the other hand, although soy protein is an oligomeric protein with many subunits, it has only four binding sites and the

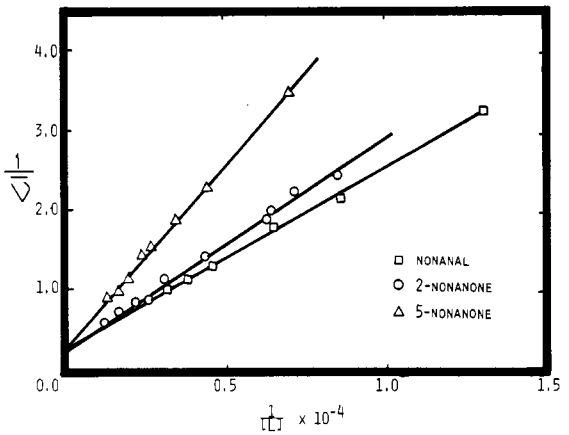


**Figure 2.** Effect of the position of the keto group in the chain on the binding affinity of ketones to soy protein. Double-reciprocal plots of binding of nonanal, 2-nonanone, and 5-nonanone to soy protein at 25 °C in 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. $\bar{\nu}$ is the number of moles of ligand bound per mole of soy protein, and [L] is the molar concentration of the free ligand.

hydrophobicity of these sites may not be as strong as the hydrophobicity of those of bovine serum albumin.

**Effect of Position of the Keto Group.** Since the binding of methyl ketones to soy protein is hydrophobic in nature, the effect of the position of the keto group in the chain was investigated. The double-reciprocal plots for the binding of nonanal, 2-nonanone, and 5-nonanone to soy protein are shown in Figure 2. The intercept in Figure 2 is the same irrespective of the position of the keto group in the chain. This suggests that the above three ligands bind to the same number of binding sites in soy protein but the binding affinity decreases as the keto group is shifted toward the middle of the chain. Nonanal, in which the keto group is in the terminal 1 position, exhibits higher binding affinity than 2-nonanone and 5-nonanone, in which the keto group is present in the second and fifth position, respectively. The linear decrease in the binding affinity as the keto group is shifted to the center of the chain (Table I) suggests that the keto group which is relatively polar provides steric hindrance to the hydrophobic interaction between the ligand and the binding site in the protein. In the case of nonanal, the presence of the keto group in the terminal 1 position permits unhindered contact between the entire length of the ligand and the binding site in the protein. With 2-nonanone and 5-nonanone, the presence of the keto group in the second and fifth position introduces steric hindrance and thus destabilizes the hydrophobic interaction. Such destabilization of the hydrophobic interaction progressively increases as the keto group is shifted to the center of the chain. For each shift in the keto group from the terminal 1 position to the center of the chain, the hydrophobic free energy of association becomes more positive by 105 cal/mol (Table I).

**Effect of Temperature.** The effect of temperature on the binding of 2-nonanone to soy protein isolate is shown in Figure 3A. The same data are presented in the form of double-reciprocal plots in Figure 3B. Temperature had very little effect on the binding constant as well as the number of binding sites at 25 and 45 °C. This is clearly reflected in the double-reciprocal plot (Figure 3B), wherein the slope and the intercept at 25 and 45 °C are identical. At 5 °C the slope and the intercept are markedly different from those at 25 and 45 °C. It has been reported that in many systems, the hydrophobic interaction between a ligand and protein is independent of temperature (Steinhardt and Reynolds, 1969; Damodaran and Kinsella, 1980). In other words, the enthalpy change for the hydrophobic association process is almost zero. This is mainly because of the fact that the hydrophobic association is a manifestation of the changes in the entropy rather than in the enthalpy of the system, and the hydrophobic free energy is derived mainly from the positive changes in the entropy and not in the enthalpy (Kauzmann, 1959). But, contrary to this fact, the binding behavior of 2-nonanone to soy protein at 5 °C is markedly different from that at 25 and 45 °C. The binding affinity at 5 °C is 2000 $M^{-1}$, whereas at 25 and 45 °C it is only 930 $M^{-1}$. The total number of binding sites decreases to two sites at 5 °C compared to four at 25 and 45 °C (Figure 3B). These data suggest that the temperature-induced changes in the binding affinity may be due to major changes in the tertiary and quaternary structures of soy protein at low temperatures. It is known that the 11S fraction of soy protein precipitates at low temperature (Briggs and Mann, 1950; Saio et al., 1968). However, the actual mechanism is not known. It may be speculated that the major interaction responsible for the quaternary structures of various soy fractions may be the hydrophobic bond. At low temperatures, the weakening of the hydrophobic interactions within these structures may result in the reorganization of the subunits, thus creating completely different hydrophobic binding sites having higher binding affinities.

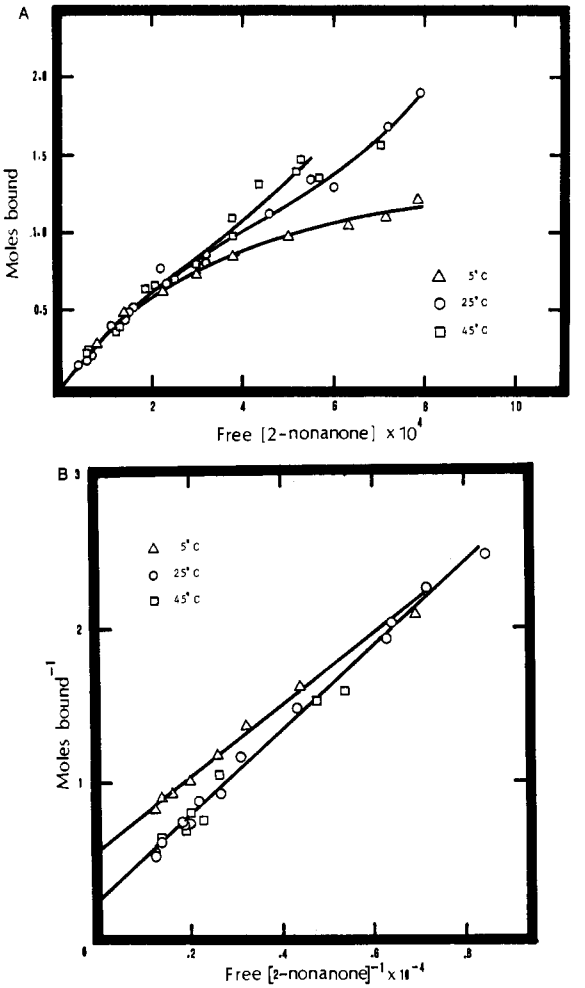


**Figure 3.** Effect of temperature on the binding of 2-nonanone to soy protein. The temperatures used were 5 (△), 25 (○), and 45 °C (□). The buffer system used was 0.03 M Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol. (B) Double-reciprocal plot of 2-nonanone binding to soy protein at 5 (△), 25 (○), and 45 °C (□).

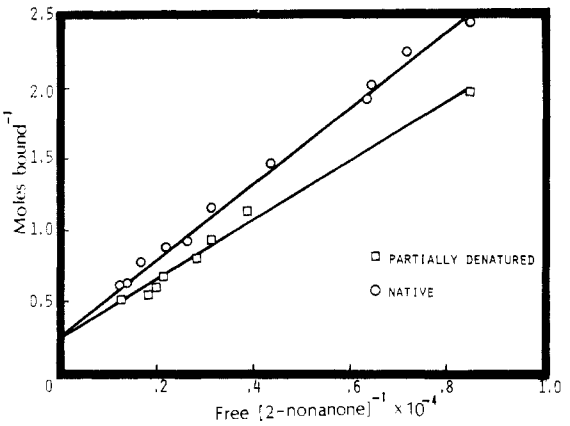


**Figure 4.** Effect of partial denaturation of soy protein on the binding of 2-nonanone at 25 °C. Soy protein was denatured by heating a 1% solution at 90 °C for 1 h. (□) Partially denatured; (○) native soy protein.

The rationale for such interpretation is that when there are no structural changes, the hydrophobic free energy of interaction between a ligand and protein should increase with temperature (since $\Delta G° = -RT \ln K$). But in the 2-nonanone–soy protein system, the free energy change at 5 °C is higher (−4.221 kcal/mol) than that at 25 °C (−4.045 kcal/mol). Since the free energy change can also be expressed as $\Delta G = \Delta H - T\Delta S$, the greater free energy change at 5 °C may be due to either greater (negative) changes in the enthalpy or positive changes in entropy of the system. Since enthalpy is directly dependent on the temperature, the enthalpy change should be less at low temperatures. Therefore, the greater negative free energy change at 5 °C for the 2-nonanone–soy protein interaction is ostensibly due to positive changes in the entropy of the system. Such positive changes in the entropy may be due to structural rearrangement of the subunits in soy protein from a more ordered state to a less ordered state at low temperatures. Such changes may also create totally new binding sites having a higher binding affinity for the ligand.

**Effect of Partial Heat Denaturation.** The binding affinity of a ligand for a protein is dependent upon the structural state of the binding sites. Any change in the native conformation of the protein, for example, heat denaturation, would profoundly affect the binding affinity. Since most of the commercial preparations of soy protein isolates are partially denatured, the effect of such partial denaturation on the binding of 2-nonanone was studied.

Partial denaturation of soy protein (heated at 90 °C for 1 h) increased the binding affinity for 2-nonanone compared to that of native soy (Figure 4). While the binding constant for native soy is 930 $M^{-1}$, the binding constant for partially heat denatured soy is 1240 $M^{-1}$. This is consistent with earlier reports that heat denaturation of soy protein increased the resistance to remove off-flavors by vacuum distillation (Arai et al., 1970). However, the double-reciprocal plots exhibited a common intercept for both native and partially denatured soy protein (Figure 4). This suggests that under the denaturation conditions used, the number of binding sites for 2-nonanone in soy protein does not change, wheras the binding affinity of the ligand to the same sites increases by about 30%. This implies that partial denaturation does not alter the oligomeric nature of soy protein to any great extent and no additional hydrophobic surfaces are created for the binding of off-flavor compounds. But, the quaternary structure of soy protein may undergo certain reorganization of the subunits which may enhance the hydrophobicity of the previously existing hydrophobic sites and thus increase the binding affinity for the ligand. Such an interpretation is consistent with the resistance of soy protein to heat denaturation.

Comparison of our results with some of the reports in the literature reveals some disparities. Beyeler and Solms (1974) derived binding constants for many flavor ligands using the equilibrium dialysis method. They obtained the binding constants from the relationship

$$\bar{r} = KC$$

where $\bar{r}$ is the number of moles of ligand bound per mole of protein, $K$ is the binding constant, and $C$ is the molar concentration of the free ligand. For example, the binding constant for 2-butanone to soy protein isolate at 20 °C and pH 7.0 was estimated to be 5174 $M^{-1}$. This value is very high when compared to the binding constant for hexanol, i.e., 173 $M^{-1}$, obtained by Arai et al. (1970) or 930 $M^{-1}$ for 2-nonanone obtained in this study. The validity of using the above equation to calculate the binding constant is questionable, since, by definition, the equilibrium binding constant is the reciprocal of the molar concentration of the free ligand at which half of the total number of binding sites are saturated and not the slope of the binding isotherm. Furthermore, assuming the molecular weight of soy protein as 50 000, Beyeler and Solms (1974) obtained molal ratios of binding up to 1200 for 2-butanone. In a 50 000 molecular weight protein, the total number of amino acid residues may be assumed to be about 450. This gives a value of about 3 mol of butanone bound to each amino acid residue. This seems very unlikely. The possible reason for this abnormal value may be the physical state of the protein aggregates. In a surface adsorption process the ligand may form multiple layers around the protein surface or get trapped in the matrix of the protein aggregates. In such cases it is very difficult to calculate the actual number of moles of ligand bound per mole of protein. Since Beyeler and Solms (1974) used insoluble protein suspensions, the binding constants derived for many flavor ligands may not reflect the molecular nature of interaction of the ligands with the protein. As mentioned earlier, if the objective is to solve the off-flavor problem in soy protein, then it is important to understand the interaction of native protein with flavor ligands.

In a different approach, using gel filtration techniques, Arai et al. (1970) studied the interaction of hexanal and 1-hexanol with partially denatured by soy protein. They obtained the binding constants 173.4 $M^{-1}$ and 80.3 $M^{-1}$ for hexanal and 1-hexanol, respectively. At saturation levels the amounts of hexanal and 1-hexanol bound to partially denatured soy protein was about 0.847 and 0.889 mg/g of protein, respectively. when the molecular weight of soy protein is assumed to be 100 000, the total number of binding sites, calculated from the above values, in partially denatured soy protein is about one. For an oligomeric protein such as soy protein, this value seems to be very low and does not agree with the results presented in this study. The agreement between the results presented here and the other theoretical and experimental predictions on the hydrophobic free energy of interaction (Wishnia, 1962, 1963; Damodaran and Kinsella, 1980) indicates that there are about four binding sites for carbonyls in the native soy protein. On the basis of the fact that the binding constant increases 3-fold for each methylene group increase in the chain, it may be speculated that the binding constant for hexanal would be about 40 $M^{-1}$. Thermodynamically this suggests that the interaction between the carbonyls and soy protein is relatively weak, and since the binding process is reversible, it should be possible to remove the off-flavors under appropriate conditions.

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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