Determination of the Binding of Hexanal to Soy Glycinin and β-Conglycinin in an Aqueous Model System Using a Headspace Technique

Sean F. O'Keefe,† Lester A. Wilson, Adoraccion P. Resurreccion,‡ and Patricia A. Murphy*

Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa 50011

The equilibrium binding of hexanal to soybean glycinin and β-conglycinin has been evaluated under several conditions. Binding of the flavor ligand was allowed to approach saturation. In 0.3 M Tris, pH 8.0, glycinin and β-conglycinin had similar affinities, 270 and 303 M^{-1}, respectively. The numbers of binding sites for hexanal for glycinin and β-conglycinin were 108 and 26, respectively. Different buffer conditions, 0.5 M NaCl with 0.02% NaN_3 or 10 mM β-mercaptoethanol, changed the number of binding sites and affinity for hexanal for these proteins. Positive cooperativity was observed for the binding of hexanal to both proteins. ANS surface hydrophobicity and turbidity increases suggested that structural changes may occur as a result of hexanal binding.

INTRODUCTION

The use of soy proteins as a functional ingredient in food products would be increased if the beany off-flavor problem is solved (Kinsella and Damodaran, 1980). Off-flavor compounds implicated include a number of volatile compounds that are formed via lipoxygenase-catalyzed oxidation of the unsaturated lipid present in soy (Wolf, 1975). The compounds include pentanol, hexanol, heptanol, hexanal, 3-*cis*-hexenal, 2-propanone, 2-pentylfuran, and ethyl vinyl ketone (Hill and Hammond, 1965; Arai et al., 1967; Cowan et al., 1973; Maga, 1973; Sessa and Rackis, 1977; Rackis et al., 1979; Hsieh et al., 1982). These compounds are associated with the proteins in soy and are not completely removed by the techniques employed in the production of soy protein concentrates and isolates. The compounds are carried into the final product and can cause unacceptable flavors. The binding of added flavors by soy proteins can cause problems in determining the appropriate level of flavoring during product formulation (Malcolmson et al., 1987).

Damodaran and Kinsella (1981a,b) have suggested that the binding of carbonyl flavor compounds with soy proteins is due entirely to hydrophobic bonding. However, the binding of carbonyl flavor compounds to soy proteins cannot be easily explained as resulting from hydrophobic bonding alone. The lack of interaction of hexane (Thissen, 1982), carboxylic acid (Beyeler and Solms, 1974), and alcohols (Gremli, 1974) is not explained. The binding parameters obtained by O'Neill and Kinsella (1987) and Damodaran and Kinsella (1981a,b) must be viewed with caution because the binding site number and constants were obtained by extrapolation of very small portions of the binding curves, because the proteins used in the earlier study were partially purified, and because 2-mercaptoethanol or sodium azide used in their studies may affect binding constants and sites. For these reasons, the binding of hexanal to purified soy proteins was examined.

* To whom correspondence should be addressed.
† Present address: Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL.
‡ Present address: International Rice Research Institute, Los Banos, Philippines.

MATERIALS AND METHODS

Purification of Soy Proteins. Vinton 81 soybeans were obtained from the Agronomy Department at Iowa State University. The beans were stored in a cold room at 5 °C in a tightly closed container until use. The beans were ground in a spice mill, and the lipid was extracted by stirring the ground beans with numerous changes of Skelly B (soy:solvent, 1:5 v/v) at room temperature until no further lipid was extracted; usually, about 10 solvent changes were necessary. The defatted soy was further ground in the spice mill and passed through a 60-mesh sieve to remove hull fragments. A crude protein fraction was prepared by using the method of Thanh and Shibasaki (1976) with the following modification. In place of two isoelectric precipitations at pH 6.4 and 4.8, three precipitations were done at pH 6.4, 5.3, and 4.8. The fraction precipitating at pH 6.4 was crude glycinin, the fraction precipitating at pH 5.3 contained a mixture of glycinin and β-conglycinin and was discarded, and the fraction precipitating at pH 4.6 was crude β-conglycinin. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed that the crude glycinin fraction was contaminated with β-conglycinin and vice versa. The precipitated fractions were resolubilized in phosphate buffer (45 mM phosphate, pH 7.4, 10 mM 2-mercaptoethanol, 0.02% sodium azide, 400 mM NaCl). The crude β-conglycinin fraction was purified by passage through Sepharose 6B-Cl twice. The crude glycinin fraction was similarly treated, and both proteins were further purified by using concanavalin A–Sepharose 4B (Kitamura et al., 1974). The β-conglycinin was eluted from the concanavalin A column with the phosphate buffer containing 0.01 M methyl D-mannoside. Fractions were collected each 30 min (13 mL), protein was determined by UV 280-nm absorption, and the fractions of interest were concentrated by using ultrafiltration with 50K Nucleopore membranes. The proteins were stored at 5 °C in concentrated solution (>40 mg/mL) in phosphate buffer containing 0.02% sodium azide and 10 mM 2-mercaptoethanol.

Before use in the binding studies, the proteins were dialyzed at 5 °C again with standard Tris-HCl buffer (pH 8.0, 30 mM) in dialysis sacs filled one-third full. The dialysis buffer was changed daily for 4 days. The protein solutions were diluted with standard Tris buffer to 12 mg/mL and placed in new dialysis sacs, and the proteins were further dialyzed for 1 day. The 24-h dialysis of diluted protein was done to ensure that the concentrated protein did not coat the inner surface of the dialysis bag and inhibit equilibrium. The protein solutions were diluted to 10.0 mg/mL with standard Tris buffer. Protein concentrations were determined by using the Biuret method with BSA standard. The duration of the dialysis procedure was 5 days to ensure that the buffer transfer was complete and that sodium azide and 2-mercaptoethanol were removed. Enough protein was processed at one time for 2 weeks of experiments. A total plate count and

0021-8561/91/1439-1022$02.50/0

psychrophile count at the end of 2 weeks of refrigerated storage indicated that bacterial growth was not a problem in the proteins (both counts were below 500 organisms/mL).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The discontinuous method of Laemmli (1970) was used. A 10–15% gradient polyacrylamide gel was prepared by using acrylic cells of equal volume. The acrylamide to bis(acrylamide) ratio was 37.5:1. The resolving and stacking gel pH values were 8.8 and 6.8, respectively. Staining and destaining of the gels were done by using 30% methanol/7% acetic acid/0.1% Coomassie Brilliant Blue R and 30% methanol/7% acetic acid, respectively.

Antibody Production and Rocket Immunoelectrophoresis. The method of Murphy and Resurreccion (1984) was used.

Syringe Calibration. A 1.0-μL syringe was obtained from Hamilton (Reno, NV). The calibration of the syringe was determined by using a dye-transfer procedure. Briefly, a 40.0 mg/mL solution of bromophenol blue dye in distilled water, pH 9.9, was serially diluted from 1:100 to 1:200 000, which represented concentrations of 0.2–400 ng/μL. The range of 0.2–40 ng/μL was determined to follow Beer's law (r^2 = 0.9999). The 1.0-μL syringe was used to transfer 0.05 and 0.10–1.0 μL, in 0.10-μL steps, of the concentrated dye solution into 3 mL of distilled water brought to pH 9.9 with dilute NaOH. This represented concentrations of 0.667–13.329 μg/mL (dilution factor included). Care was taken to wipe solution from the needle without wicking. The absorbance at 540 nm was determined for the serial dilutions and the syringe-transferred solutions. The theoretical and observed absorbances were compared to determine the actual volume of dye solution transferred. The 95% confidence limits for four independent replications were determined. The syringe calibration indicated that the 95% confidence limit for the actual amount of dye transferred encompassed the theoretical value from 0.05 to 0.60 μL. Above 0.60 μL, the theoretical amount was lower than the 95% confidence limit of the actual amount. The percentage of the theoretical value actually transferred above 0.60 μL ranged from 102 to 104%. These correction factors were used in the data analysis. The coefficient of variation (COV) of four replications was below 1.3% for all volumes transferred except 0.05 μL, for which it was greater than 5%; therefore, this level of transfer was not used. The regression of COV against level of dye transferred from 0.10 to 1.0 μL was not significantly different from zero.

Determination of Binding. Reaction vials of 2-mL volume with caps and Teflon-lined septa were purchased from Supelco (Supelco Park, PA). Small stirring bars were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ). The protein solutions were equilibrated at 20 °C before 1.00 mL was loaded into the reaction vials. For ligand concentrations ranging from 100 to 1000 ppm of hexanal (Aldrich Chemical Co., Milwaukee, WI), the corresponding volume of ligand was carefully added to the reaction tube, and the tube was tightly capped. The additional volume added was ignored in calculations. For concentrations from 1 to 50 ppm, a 100 ppm solution of hexanal in buffer was prepared by adding 10 μL of hexanal to buffer in a 100-mL volumetric flask previously brought to volume and containing an added stir bar (Buttery et al., 1969). The volumetric flask was chosen to have a minimum headspace area. The solution was stirred overnight. The protein solution (0.5 mL, 20 mg/mL) was loaded into a vial, and a combination of buffer and ligand-containing buffer was added (0.5 mL) to produce the desired concentration. The ligand solution was added last, and new solutions were prepared for each of three replications.

The equilibration time was determined for hexanal at concentrations of 5, 100, and 500 ppm. The headspace concentrations of the protein solutions and control, containing buffer but no protein, were determined after equilibration by using gas chromatography (GC). A 100-μL gastight syringe with replaceable plunger tip was obtained from Hamilton. The syringe was kept at 35 °C between injections and was cleaned by using a heated, vacuum syringe cleaner (Hamilton). The headspace was sampled by withdrawing 100 μL of headspace gas and immediately injecting onto the GC. The vial was sampled two more times where 100 μL of ligand-free gas was injected into the vial to prevent vacuum formation. The plunger tip was replaced at least once daily to ensure reliability. A Varian Model 2400 (Sunnyvale, CA) gas chromatograph was equipped with a flame ionization detector, and a bonded DB-1 Megabore column (30 m × 0.72 mm i.d., J&W Scientific Inc., Rancho Cordova, CA) was held isothermally at 100 °C. The gas flow rates were 15.5, 30, and 250 mL/min for nitrogen, hydrogen, and air, respectively. Peak quantitation was done with a Beckman 427 plotting integrator. The detector and injector temperatures were held at 150 °C.

The protein molarity was calculated from the protein concentration by using molecular weights of 160 000 and 320 000 for β-conglycinin and glycinin, respectively (O'Neill and Kinsella, 1987).

The amount of bound ligand was determined by using the equation [bound] = $((B - P)/B)[T]$, where B is the headspace concentration for the buffer blank, P is the headspace concentration for the protein buffer solution, and T is the total ligand concentration.

Effect of Reducing Agent, Ionic Strength, and Azide on Binding. The binding with and without 10 mM 2-mercaptoethanol, 0.5 M NaCl, and sodium azide (0.02%) was determined. The solutions were prepared to give these concentrations by addition of the reagents to the buffer and protein solutions maintaining a 10 mg/mL protein level.

Determination of Protein Surface Hydrophobicity and Reactive Lysine. The method reported by Kamata et al. (1984) was used to determine hydrophobicity. A final volume of 4 mL was prepared to contain 8 mg of protein, 125 μM 1,8-anilinonaphthalosulfonic acid (ANS), and 30 mM Tris buffer, pH 8.0. The tubes were incubated for 3 h. The fluorescent emission was recorded at 475 nm after excitation at 395 nm. The hydrophobicity of glycinin and that of β-conglycinin were determined after reaction with hexanal at five levels: 0, 5, 100, 500, and 1000 ppm. The hydrophobicity was determined for glycinin and β-conglycinin before and after the proteins were heated at 100 °C for 4 min to determine if surface hydrophobicity increased with protein unfolding. Experiments were done in duplicate.

The dye binding method of Perl et al. (1985) was used to determine reactive lysine with the following modification. A buffered protein solution (3 mL of 50 mg/mL) was used instead of dry protein. The reacted solutions were filtered through a 0.45-μM filter before dilution and absorbance reading. The results were calculated as change in Orange G absorbance in samples reacted with ligand, 50 and 500 ppm hexanal, and unreacted protein.

Extraction of Bound Lipid and Determination of Lipid Classes. All solvents were of reagent grade and were redistilled in glass before use. About 200 mg (5 mL) of the isolated soy proteins was extracted with chloroform/methanol (50 mL, 2:1 v/v) by using a blender. The emulsions that formed were allowed to separate overnight in separatory funnels. The organic phases were filtered through Celite, and the solvent was removed under vacuum. The lipid obtained was dissolved in 20 μL of chloroform. The lipid classes triglyceride, free fatty acid, and polar lipid were determined by using the thin-layer chromatography flame ionization Iatroscan system (Innis and Clandinin, 1981). The rods were spotted with 1 μL of the extracted lipid. Standard phosphatidylcholine and stearate were obtained from Sigma (St. Louis, MO). Corn oil was used as a triglyceride standard. About 10 μg of each standard was spotted per rod. The solvent system was hexane/diethyl ether/acetic acid (97:3:1 v/v).

RESULTS AND DISCUSSION

Protein Purity. The SDS–PAGE showed that the protein preparations were very pure. Only very minor contaminating bands were observed even at high protein loads (Figure 1). Modification of the Thanh and Shibasaki isoelectric precipitation procedure increased the purity of the crude β-conglycinin fraction, but the yield was much lower. The immunoelectrophoresis indicated that there was no immunoreactive glycinin in the β-conglycinin preparation, and the glycinin preparation contained only 0.06% β-conglycinin. The protein load used to determine extremely small amounts of cross contamination was 100 μg. Under the conditions employed, the minimum detectable amount of protein was about 25 ng.

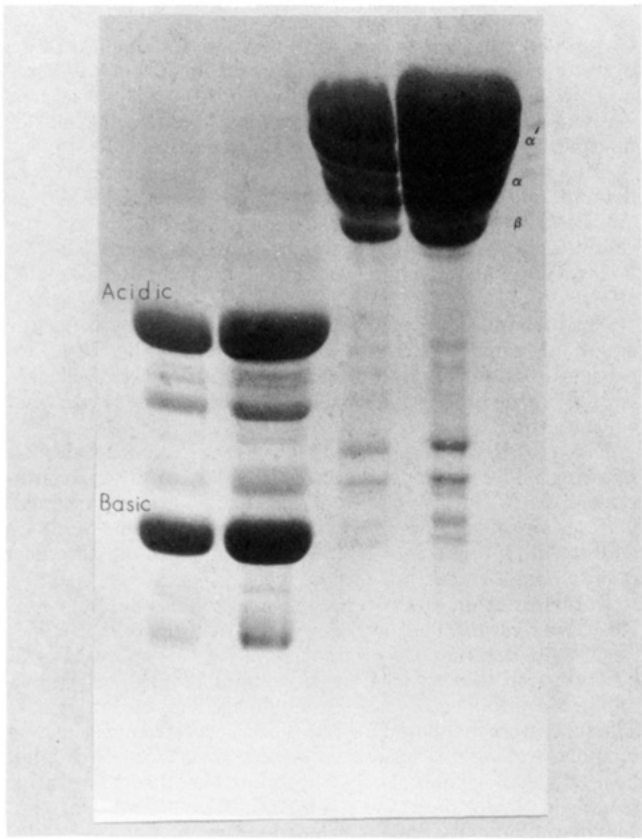

Figure 1. Polyacrylamide electrophoresis of purified soybean glycinin (left lanes) and β-conglycinin (right lanes); 100 μg/lane.

Lipid Bound to Purified Proteins. The Iatroscan analysis of the extracted lipid indicated that polar lipid predominated, although trace amounts of triglycerides and free fatty acid also were present. The estimated quantities of polar lipid were 2.23 and 1.49 mg/g for glycinin and β-conglycinin, respectively. The minor amount of polar lipid bound to the protein would likely be removed completely only by use of solvents more polar than alkanes. The effect of bound lipid on ligand binding would be difficult to establish because removal with polar solvents would likely involve protein denaturation. An unambiguous identification of what polar lipid classes are involved would allow the study of the effects of addition of small amounts of lipid to the protein and of the effects of this binding on ligand–protein interactions.

Binding of Hexanal to Glycinin and β-Conglycinin. The linear regression of the repeated sampling of the headspace vials revealed no significant change in headspace concentration over five injections at 50, 500, and 1000 ppm of hexanal ($P < 0.05$). This indicated that the amount of dilution taking place did not affect the headspace concentration. For both buffer and water, the headspace hexanal at equilibrium was linear with total added hexanal. This demonstrates that saturation of the aqueous phase did not take place. All of the hexanal at the concentrations studied was solubilized, and no emulsion droplets formed.

The binding curve obtained for each protein at 30 °C is illustrated in Figure 2A. Saturation of the binding sites seems to have been obtained. The double-reciprocal plot of these data is shown in Figure 2B. The curvature of the double-reciprocal plot indicates that positive cooperativity may be present. The modified double-reciprocal plot can indicate the minimum number of classes of sites involved in the cooperative effect (Thissen, 1982; Segal, 1975). In this modification, the reciprocal of the free ligand concentration is brought to different powers; the power

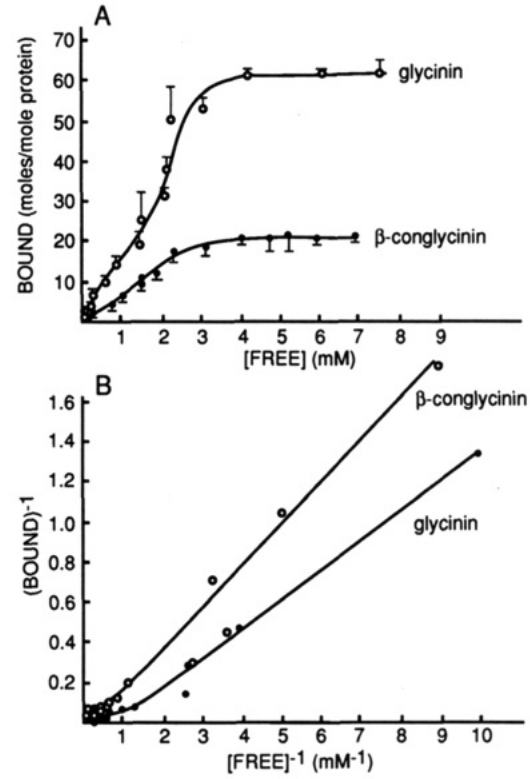

Figure 2. Binding of hexanal to glycinin and β-conglycinin. (A) Saturation curve. (B) Double-reciprocal plot.

Table I. Binding Parameters for Hexanal to Glycinin and β-Conglycinin Reacted under Different Conditions

protein and condition	graph fit		Klotz plot		Wilkinson (1961)	
	n	K, M^{-1}	n	K, M^{-1}	n	K, M^{-1}
glycinin						
0.03 M Tris	60	555	84	483	108 ± 10	270 ± 24
0.5 M NaCl	33	200	43	172	38 ± 3	238 ± 32
0.02% NaN_3	77	1075	77	1172	76 ± 3	1250 ± 183
10 mM BME	62	500	122	180	112 ± 10	213 ± 24
β-conglycinin						
0.03 M Tris	21	175	23	1437	26 ± 10	303 ± 30
0.5 M NaCl	22	500	26	1431	68 ± 8	125 ± 14
0.02% NaN_3	29	500	40	255	32 ± 2	417 ± 83
10 mM BME	20	625	36	235	32 ± 2	303 ± 33

that produces a straight line is the apparent (minimum) number of cooperating classes of sites. Segal (1975) pointed out that this number is not necessarily an integer and is a minimum number because strong cooperativity is assumed. The correlation coefficients, obtained for different powers, indicated that the apparent number was about 1.3 for β-conglycinin and 1.5 for glycinin. That the numbers are not integers probably indicates that there are two classes, but the cooperativity is not high.

The numbers of binding sites per protein molecule can be estimated from the saturation plots to be about 60 and 21 for glycinin and β-conglycinin, respectively. The double-reciprocal plot binding constant and number of sites were quite different as determined by linear regression using double reciprocal and by the method of Wilkinson (1961) and are illustrated in Table I. The number of binding sites obtained from the Wilkinson procedure was higher than estimated from the saturation plots: 108 ± 10 and 26 ± 10 for glycinin and β-conglycinin, respectively. Because of the curvature of the double-reciprocal plot, the parameters could not be estimated from least-squares regression because of the negative Y intercept obtained. The affinity constants obtained indicate that the affinity for hexanal was slightly greater for β-conglycinin than for

glycinin: 303 ± 30 and 270 ± 24 M^{-1}, respectively. A Student's t-test using a pooled variance indicated that there was no significant difference between these affinity constants ($P < 0.05$).

The association of volatile carbonyl compounds with aqueous solutions of soy proteins has been studied by a number of authors. Arai et al. (1970) have reported that the binding constant was greater for hexanal than for hexanol, but the maximum number of binding sites that can be estimated from their data was about 1.7 for both compounds. Gremli (1974) looked at the retention of a series of carbonyl flavor compounds by a 5% solution of soy protein. The retention of aldehydes increased as the chain length increased, and unsaturated aldehydes were retained to a greater extent than the corresponding saturated compounds. Aldehydes were retained to a greater extent than corresponding ketones. Alcohols were found to have no affinity for the protein. The numbers of binding sites that can be estimated from the data presented by Gremli (O'Keefe, 1988) are >27 and >8 for heptanal and 2-nonanone, respectively.

Solms et al. (1973) and Beyeler and Solms (1974) examined the binding of compounds including butanal and 2-butanone to a soy protein isolate. The maximum molal binding values that can be estimated from the presented curves, by using a realistic molecular weight estimate of 220 000, seem to be 80 and 270 for butanal and 2-butanone, respectively. Solms et al. (1973) suggest that the binding of ligands to hydrophobic regions of soy proteins causes protein unfolding, with the creation of new binding sites.

Damodaran and Kinsella (1981a,b) investigated the binding of carbonyls to soy proteins. An increase in the binding constant as chain length increased for 2-ketones was given as evidence that the binding was simply hydrophobic. The binding curves presented did not seem to show saturation and, therefore, suggest that the number of binding sites could have been, at least, twice the greatest number observed, that is, higher than 4, 7, and 10 for 2-nonanone, 2-octanone, and 2-heptanone, respectively. However, the authors suggested that there were only 4–5 binding sites from the double-reciprocal (Klotz) transformation. The molecular weight used in calculations was 100 000, which is incorrect. The binding constant for 2-nonanone was higher at 5 °C than at 25 and 45 °C (2000 compared with 950 M^{-1}). Using semipurified proteins obtained from the method of Thanh and Shibasaki (1976), Damodaran and Kinsella (1981a,b) reported that the binding constant for 2-nonanone with β-conglycinin was much greater than that with glycinin. However, the binding curves indicated that glycinin had a sigmoidal rather than typical hyperbolic pattern. This suggests that positive cooperation was present. Increasing the ionic strength of a glycinin solution to 0.5 N caused an increase in affinity, and a binding constant of 290 M^{-1} with 8 binding sites was reported.

Thissen (1982) and Wilson (1986) investigated the use of a headspace technique to determine binding parameters for a series of aldehydes, ketones, hexanal, and hexane with a soy protein isolate. The binding curves indicated that positive cooperativity was present, and the modified double-reciprocal plot indicated that at least three different cooperating sites were present. There was a clear effect of functional group because hexane was found to have no affinity. The number of binding sites for ketones was higher than reported by Damodaran and Kinsella; 50, 82, 50, and 15 sites were found for C5, C6, C7, and C9 2-ketones, respectively. Thissen (1982) and Wilson (1986) pointed out that the methods used to calculate the binding constant gave greatly different results; for 2-hexanone, the binding constants at 25 °C were 33, 1471, 146, and 290 M^{-1} calculated by using the methods of Damodaran and Kinsella (1981a), Beyeler and Solms (1974), Arai et al. (1970), and the modified Klotz procedure, respectively.

O'Neill and Kinsella (1987) examined the binding of 2-nonanone to purified soy glycinin and β-conglycinin. The protein purification involved a combination of the techniques of Thanh and Shibasaki (1976) and Kitamura et al. (1974). The numbers of binding sites were reported to be 2.8 and 9.9, and the binding constants were 3136 and 539 M^{-1} for β-conglycinin and glycinin, respectively, when correct molecular weights were used. The difference in results obtained by O'Neill and Kinsella (1987) and Damodaran and Kinsella (1981b) was attributed to the use of incorrect absorptions of a 1% solution at 280 nm in the earlier work. The binding curve illustrated for glycinin indicated that saturation was not approached. The range of moles bound examined for glycinin was only 0.3–1.0. If the number of binding sites was truly 9.9, Damodaran and Kinsella (1981b) and O'Neill and Kinsella (1987) investigated less than 10% of the binding curve. Extrapolation of a small fraction of a binding curve to obtain binding parameters has been criticized (Klotz, 1982, 1986; Klotz and Hunston, 1975, 1979; Connors, 1987). The proteins used by Kinsella et al. were freeze-dried for storage. Freeze-drying has been reported to decrease the sulfhydryl content of glycinin (Hoshi and Yamauchi, 1983) and may result in irreversible insolubilization of some of the protein (Fukushima, 1980), including β-conglycinin (Hoshi et al., 1982). The effect of these structural changes on ligand binding is unknown.

There was no measurable absorbance at 540 nm up to 300–400 ppm of hexanal in the protein solutions. The soy protein solutions showed some development of turbidity above 300 ppm of hexanal for glycinin and above 400 ppm for β-conglycinin during the 15–18 h used to bring the system into equilibrium. The immunoreactivity of the two proteins did not change at any hexanal concentration used in our binding studies nor at 10 times the higher concentration of hexanal reported here. Precipitates could not be centrifuged out to estimate how much protein contributed to the turbidity. We believe the amounts to be extremely low. The turbidity for β-conglycinin was never more than 0.1 at 540 nm nor greater than 0.4 for glycinin at 1000 ppm of hexanal. All other concentrations of hexanal produced much lower absorbance values.

The surface hydrophobicity was determined before and after the purified protein solutions were heated on a boiling water bath for 4 min. The increase in hydrophobicity was much greater for glycinin than for β-conglycinin; the ratios of fluorescent intensity after/before heat treatment were 4.6 ± 0.1 and 2.1 ± 0.1, respectively. This agrees with Guzman (1984), Yamagishi et al. (1983), and Iwabuchi and Shibasaki (1981a,b), who found that the β-conglycinin structure was much more heat stable than glycinin in a highly purified system devoid of reducing agent as determined by immunoelectrophoresis. However, contrasting results were obtained by using differential scanning calorimetry by Marshall and Zarins (1989). The surface hydrophobicity of glycinin and β-conglycinin increased as the level of hexanal increased ($P < 0.05$).

The decreases in absorbance of Orange G, indicating loss of reactive lysine, after reaction with hexanal at 50 and 500 ppm were very minor: glycinin 0.018 ± 0.006 and 0.015 ± 0.003, β-conglycinin 0.038 ± 0.009 and 0.025 ± 0.009, respectively. There were no significant differences

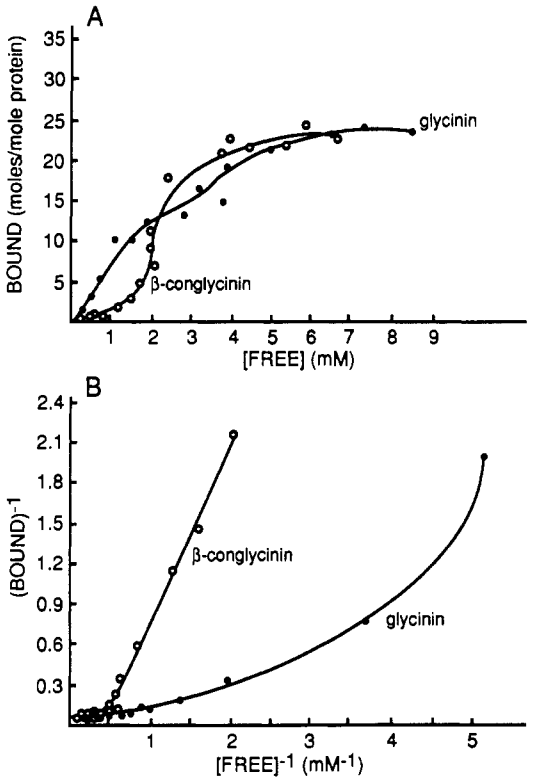

Figure 3. Binding of hexanal to glycinin and β-conglycinin in model system containing 0.5 M NaCl. (A) Saturation curve. (B) Double-reciprocal plot.

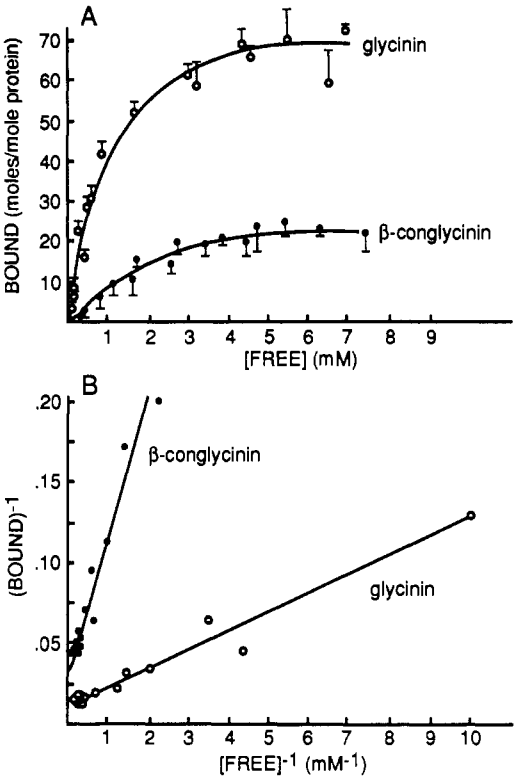

Figure 4. Binding of hexanal to glycinin and β-conglycinin in a model system containing 0.02% sodium azide. (A) Binding curve. (B) Double-reciprocal plot.

between ligand level or between proteins as determined by a Student's *t*-test with pooled variance. The changes in absorbance were greater for glycinin than for β-conglycinin, but the changes were small, ranging from 0.013 to 0.045. However, a Student's *t*-test indicated that the observed absorbances were significantly different from 0 ($P < 0.05$). Perl et al. (1985) reported that the sensitivity of this method was sufficient to detect a 5% change in reactive lysine but did not indicate the absorbance change that would be expected from such a change.

The binding of hexanal to the proteins in the presence of 0.5 M NaCl is illustrated in Figure 3. The salt increased the headspace concentration in the buffer as well as in the protein tubes. The results from the Wilkinson procedure are shown in Table I and indicate that the presence of NaCl decreased the affinity of hexanal for β-conglycinin but had no significant effect for glycinin. The number of binding sites decreased to 38 ± 3 for glycinin but increased for β-conglycinin to 68 ± 8. The saturation plot (Figure 3A) seems to indicate that the number of binding sites is similar for the two soy proteins because saturation seems to have been obtained around 22–23 mol bound/mol of protein. The double-reciprocal plot (Figure 3B) illustrates that the curvature in the glycinin and β-conglycinin plots is enhanced in the presence of NaCl as compared with Figure 2B. Damodaran and Kinsella (1981a) reported that, in a model system containing 0.5 M NaCl, glycinin had 8 binding sites with a binding constant of 290 M^{-1} on the basis of 100 000 molecular weight. The number of sites would be about 26 if the correct molecular weight were used. The modified Klotz procedure indicated that a minimum of 1.5 classes were present for both proteins. Again, this possibly is due to two classes having weak cooperativity. The soy proteins are known to undergo structural changes as the ionic strength is increased from 0.03 to 0.5 N. At the higher ionic strength, glycinin is expected to exist as a dodecamer "dimer" and β-conglycinin as a trimer "monomer". At the lower ionic strength, glycinin exists as a hexamer "monomer" and β-conglycinin as a hexamer "dimer". These changes may indicate that binding sites are exposed on the protein surfaces at lower ionic strength but are masked when the proteins are assembled in mature quaternary structure.

The binding curves in the presence of 0.02% NaN_3 are illustrated in Figure 4. Comparison of the saturation curves with those obtained in buffer alone illustrates that the binding for β-conglycinin differed very little, whereas for glycinin the binding increased more rapidly and appeared to reach saturation at a higher level in the presence of sodium azide. The binding constants obtained, 1250 and 417 M^{-1} for glycinin and β-conglycinin, respectively, are higher than those for buffer alone, whereas the number of binding sites decreased for glycinin but not β-conglycinin. The double-reciprocal plots did not display the curvature observed in the absence of sodium azide. This could be because of data for binding at low ligand concentrations. If the data from 150 to 1000 ppm were examined, there would be no curvature in the double-reciprocal plots. In studies in which only a small portion of the binding curve is examined, the curvature in the double-reciprocal plot may be missed. The reason is unknown for the increased binding affinity for glycinin in the presence of sodium azide. The results are surprising because Damodaran and Kinsella (1981a) reported a low affinity of 2-nonanone to glycinin in the presence of sodium azide.

The binding of hexanal to soy proteins in the presence of 2-mercaptoethanol is illustrated in Figure 5, and the binding parameters are presented in Table I. The number of binding sites and binding constants were not significantly different for either protein, compared with the model system without mercaptoethanol. The mildly reducing conditions evidently had no effect on the binding.

The binding of hexanal to glycinin and β-conglycinin as determined in this study differs from results for

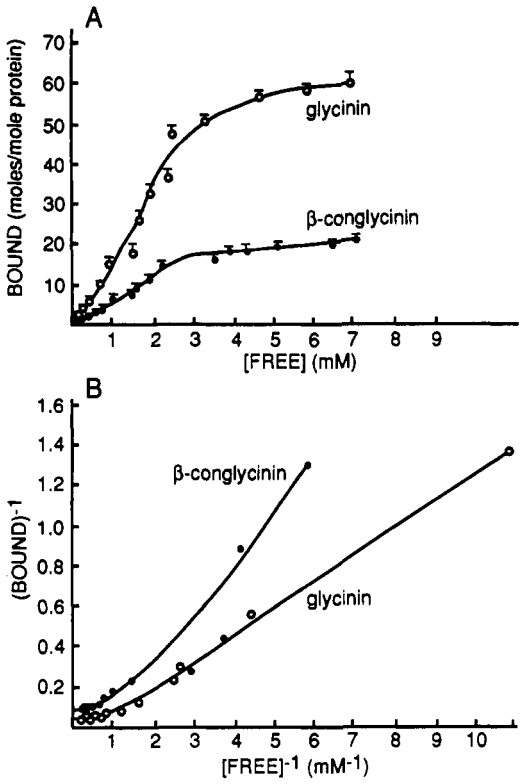

Figure 5. Binding of hexanal to glycinin and β-conglycinin in a model system containing 10 mM 2-mercaptoethanol. (A) Binding curve. (B) Double-reciprocal plot.

2-nonanone presented by Damodaran and Kinsella (1981b) and O'Neill and Kinsella (1987). The numbers of binding sites determined, 108 and 26 for glycinin and β-conglycinin, were much higher than reported by Kinsella et al. but are closer to the 81 sites reported for soy isolate by Thissen (1982) and Wilson (1986) and the estimated minimum of 27 binding sites for heptanal binding to soy isolates calculated from the data presented by Gremli (O'Keefe, 1988). The data of Arai et al. (1970) indicate, however, that fewer than 2 binding sites were present in the soy protein that was studied. The differences in the data may be due to a number of factors. The soy proteins came from different sources, have undergone different treatments, and were of different purities. Sodium azide was used and has been shown to affect the binding parameters. Partial binding curves were used to obtain data. Finally, the different techniques employed to determine binding parameters may have some role in the reported differences.

The reason for the effect of NaCl on binding could be due to the known effect that NaCl has on the tertiary structure of the multimeric soy proteins, as suggested by Damodaran and Kinsella (1981a), and/or the effect of the salt on ligand binding. Salt would be expected to increase the strength of hydrophobic bonds, but a decrease in binding constant was observed for β-conglycinin. It perhaps is necessary to investigate smaller changes in ionic strength, which would not have as great an effect on the protein tertiary structure, to determine if increasing salt concentration affects the hexanal binding in the absence of protein structural changes.

The ANS hydrophobicity and turbidity measurements indicate that structural changes occur as a result of hexanal binding. The positive cooperativity observed for binding indicates that the structural changes may affect the binding process. The small decreases in Orange G reactive lysine were not striking, but they illustrate that there may be covalent binding occurring between the aldehyde group and the lysine amino. Facile covalent binding of hexanal to protein has been reported by Okitani et al. (1986). A more sensitive test is necessary to confirm this.

It would be an oversimplification to suggest that we have a good understanding of carbonyl protein interactions. The binding is assumed to be reversible for our data calculations as is true for other studies (Wilson, 1985, 1986; Arai et al., 1967, 1970; Beyeler and Solms, 1974). Reversible binding, however, has not been proven. Because we found small changes in turbidity of the protein solutions, a true irreversible equilibrium may not have been obtained.

ACKNOWLEDGMENT

This project was supported by the Iowa Agriculture and Home Economics Experiment Station and the Iowa Soybean Promotion Board. The paper is published as Journal Paper J-13622 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Projects 2433 and 2164, the latter a contributing project to the North Central Regional Research Project, NC-136.

LITERATURE CITED

Arai, S.; Koyanagi, O.; Fujimaki, M. Studies on Flavor Compounds in Soybean. *Agric. Biol. Chem.* **1967**, *31*, 868–873.

Arai, S.; Nogushi, M.; Yamashita, M.; Kato, H.; Fujimaka, M. Studies on Flavor Components in Soybean. VI. Some Evidence for Occurrence of Protein-Flavor Binding. *Agric. Biol. Chem.* **1970**, *34*, 1569–1573.

Beyeler, M.; Solms, J. Interaction of Flavor Model Compounds with Soy Protein and Bovine Serum Albumin. *Lebensm.-Wiss. Technol.* **1974**, *7*, 217–219.

Buttery, R. G.; Ling, L. C.; Guadagni, D. G. Food Volatiles. Volatilities of Aldehydes, Ketones, and Esters in Dilute Water Solution. *J. Agric. Food Chem.* **1969**, *17*, 385–389.

Connors, K. A. Binding Constants. In *Binding Constants: The Measurement of Molecular Complex Stability*; Wiley and Sons: New York, 1987; pp 21–101.

Cowan, J. C.; Rackis, J. J.; Wolf, W. J. Soybean Protein Flavor Components: A Review. *J. Am. Oil Chem. Soc.* **1973**, *50*, 426A–435A.

Damodaran, S.; Kinsella, J. E. Interaction of Carbonyls with Soy Proteins: Thermodynamic Effects. *J. Agric. Food Chem.* **1981a**, *29*, 1249–1253.

Damodaran, S.; Kinsella, J. E. Interaction of Carbonyls with Soy Protein: Conformational Effects. *J. Agric. Food Chem.* **1981b**, *29*, 1253–1257.

Fukushima, D. Deteriorative Changes of Proteins During Soybean Food Processing and Their Use in Food. In *Chemical Deterioration of Proteins*; Whitaker, J. R., Fujimaki, M., Eds.; ACS Symposium Series 123; American Chemical Society: Washington, DC, 1980; pp 1–23.

Gremli, H. A. Interaction of Flavor Compounds with Soy Proteins. *J. Am. Oil Chem. Soc.* **1974**, *51*, 95A–97A.

Guzman, G. J. Analysis of the Effect of Ionic Strength on Thermal Denaturation of Soybean Glycinin and β-conglycinin. M.S. Thesis, Iowa State University, 1984, 96 pp.

Hill, F. D.; Hammond, E. G. Studies on the Flavor of Autoxidized Soybean Oil. *J. Am. Oil Chem. Soc.* **1965**, *42*, 1148–1150.

Hoshi, Y.; Yamauchi, F. Determination of Sulfhydryl and Disulfide Contents of Soybean 11S Globulin and Their Change by Lyophilization. *Agric. Biol. Chem.* **1983**, *47*, 2435–2440.

Hoshi, Y.; Yamauchi, F.; Shibasaki, K. On the Role of Disulfide Bonds in Polymerization of Soybean 7S Globulin During Storage. *Agric. Biol. Chem.* **1982**, *46*, 2803–2807.

Hsieh, O. A. L.; Huang, A. S.; Chang, S. S. Isolation and Identification of Objectional Volatile Flavor Compounds in Defatted Soybean Flour. *J. Food Chem.* **1982**, *47*, 16–18.

Innis, S. M.; Clandinin, M. T. Separation of Phospholipids by the TLC-FID Iatroscan System. *J. Chromatogr.* **1981**, *205*, 490–494.

Iwabuchi, S.; Shibasaki, K. Immunochemical Studies of the Effect of Ionic Strength on Thermal Denaturation of Soybean 11S Globulin. *Agric. Biol. Chem.* **1981a**, *45*, 285–293.

Iwabuchi, S.; Shibasaki, K. Immunochemical Studies of the Effect of Ionic Strength on Thermal Denaturation of Soybean 7S Globulin. *Agric. Biol. Chem.* **1981b**, *45*, 1365–1371.

Kamata, Y.; Ochiai, K.; Yamauchi, F. Relationship Between the Properties of Emulsion Systems Stabilized by Soy Protein Digests and the Protein Conformation. *Agric. Biol. Chem.* **1984**, *48*, 1147–1152.

Kinsella, J. E.; Damodaran, S. Flavor Problems in Soy Proteins: Origin, Nature, Control and Binding Phenomenon. In *The Analysis and Control of Less Desirable Flavors in Foods and Beverages*; Charalambous, G., Ed.; Academic Press: New York, 1980.

Kitamura, K.; Okubo, K.; Shibasaki, K. The Purification of Soybean 11S Globulin with Con A-Sepharose 4B and Sepharose 6B. *Agric. Biol. Chem.* **1974**, *38*, 1083–1085.

Klotz, I. M. Numbers of Receptor Sites from Scatchard Graphs: Facts and Fantasies. *Science* **1982**, *217*, 1247–1249.

Klotz, I. M. *Introduction to Biomolecular Energetics Including Ligand Receptor Interactions*; Academic Press: New York, 1986.

Klotz, I. M.; Hunston, D. L. Interactions with Small Molecules. Relationship Between Stochiometric Binding Constants, Site Binding Constants and Empirical Binding Constants. *J. Biol. Chem.* **1975**, *250*, 3001–3009.

Klotz, I. M.; Hunston, D. L. Protein Affinities for Small Molecules: Conceptions and Misconceptions. *Arch. Biochem. Biophys.* **1979**, *193*, 314–328.

Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Maga, J. A. A Review of Flavor Investigations Associated with the Soy Products Raw Soybeans, Defatted Flakes and Flours, and Isolates. *J. Agric. Food Chem.* **1973**, *21*, 864–868.

Malcolmson, J. L.; McDaniel, M. R.; Hoehn, E. Flavor Protein Interactions in a Formulated Soup Containing Flavored Soy Protein. *Can. Inst. Food Sci. Technol. J.* **1987**, *20*, 229–235.

Marshall, W. E.; Zarins, Z. M. Exothermic Transitions of Glycinin by Differential Scanning Calorimetry. *J. Agric. Food Chem.* **1989**, *37*, 869–873.

Murphy, P. A.; Resurreccion, A. Varietal and Environmental Differences in Soybean Glycinin and β-conglycinin. *J. Agric. Food Chem.* **1984**, *32*, 911–915.

O'Keefe, S. F. Binding of Volatile Flavor Compounds to Purified Soy Proteins in an Aqueous Model System. Ph.D. Dissertation, Iowa State University, Ames, 1988, 151 pp.

Okitani, A.; Kaneko, S.; Tashiro, Y.; Hayase, F.; Kato, H. Polymerization of Proteins and Impairment of Their Amino Acid Residues Due to Vaporized Hexanal. *Dev. Food Sci.* **1986**, *13*, 125–134.

O'Neill, T. E.; Kinsella, J. E. Flavor Protein Interactions: Characteristics of 2-Nonanone Binding to Isolated Soy Protein Fractions. *J. Food Sci.* **1987**, *52*, 98–101.

Perl, I. M.; Szakacs, M. P.; Kovago, A.; Petroczy, J. Stoichiometric Dye-Binding Procedure for the Determination of the Reactive Lysine Content of Soya Bean Protein. *Food Chem.* **1985**, *16*, 163–174.

Rackis, J. J.; Sessa, D. J.; Honig, D. H. Flavor Problems of Vegetable Food Proteins. *J. Am. Oil Chem. Soc.* **1979**, *56*, 262–271.

Segal, I. H. Lineweaver-Burk Plot for Allosteric Enzymes. In *Enzyme kinetics*; Segal, I. H., Ed.; Wiley and Sons: New York, 1975; pp 365–371.

Sessa, D. J.; Rackis, J. J. Lipid-Derived Flavors of Legume Protein Products. *J. Am. Oil Chem. Soc.* **1977**, *54*, 468–473.

Solms, J.; Osman-Ismail, F.; Beyeler, M. The Interaction of Volatiles with Food Components. *Can. Inst. Food Sci. Technol. J.* **1973**, *6*, A10–A16.

Thanh, V. H.; Shibasaki, K. Major Proteins of Soybean Seeds. A Straightforward Fractionation and Their Characterization. *J. Agric. Food Chem.* **1976**, *24*, 1117–1121.

Thissen, J. A. Interaction of Off-Flavor Compounds with Soy Protein Isolate in Aqueous Systems: Effects of Chain Length, Functional Group and Temperature. M.S. Thesis, Iowa State University, Ames, 1982, 119 pp.

Wilkinson, G. N. Statistical Estimations in Enzyme Kinetics. *Biochem. J.* **1961**, *80*, 324–332.

Wilson, L. A. Flavor Binding and the Removal of Flavors from Soybean Protein. In *World Soybean Research Conference III: Proceedings*; Shibles, R., Ed.; Westview Press: Boulder, CO, 1985; pp 158–165.

Wilson, L. A. Kinetics of Flavor Changes in Foods. In *Physical and Chemical Properties of Food*; Okos, M. R., Ed.; American Society of Agricultural Engineers: St. Joseph, MI, 1986; pp 382–407.

Wolf, W. J. Lipoxygenase and Flavor of Soybean Protein Products. *J. Agric. Food Chem.* **1975**, *23*, 136–141.

Yamagishi, T.; Miyakawa, A.; Noda, N.; Yamauchi, F. Isolation and Electrophoretic Analysis of Heat-Induced Products of Mixed Soybean 7S and 11S Globulins. *Agric. Biol. Chem.* **1983**, *47*, 1229–1237.

Received for review October 30, 1989. Revised manuscript received December 17, 1990. Accepted January 14, 1991.

Registry No. $H_3C(CH_2)_4CHO$, 66-25-1; NaCl, 7647-14-5.