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Binding of 14C-heptanal to soy proteins glycinin and [beta]-conglycinin

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Binding of ^{14}C-heptanal to soy proteins glycinin and β-conglycinin

by

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CHAPTER 1. INTRODUCTION

Review of Previous Work

Soy proteins

Soybean seeds have two main storage proteins, glycinin and β-conglycinin. Glycinin has a molecular weight of 360 kDa and a sedimentation coefficient of 12S. In contrast β-conglycinin is a trimer of molecular weight 180 kDa and sedimentation coefficient 7S. The acid-precipitable fraction of soybean seed proteins (at pH 4.8) is composed of 34% glycinin, 27% β-conglycinin and 39% remainder (Iwabuchi and Yamauchi, 1987). Another protein present in soybeans is the enzyme lipoxygenase (EC 1.13.11.12), which is found in quantities as high as 1% of the total protein (Kitamura, 1984).

Glycinin Glycinin is the predominant storage protein in soybean seeds. It accounts for more than 20% of the seed dry weight (Nielsen et al., 1989) in some cultivars, has no known catalytic activity, and is thought to function as a reserve for carbon and nitrogen to be used upon seed germination (Spencer, 1984). It is devoid of sugar, and does not undergo an ionic strength dependent association-dissociation phenomena. According to the current understanding, it is a hexamer with subunits situated at the vertices of a trigonal antiprism. These subunits form

two threefold rings, that are superimposed and twisted by 60° relative to one another (Plietz et al., 1987). Glycinin, like other 11S seed storage proteins, is translated as a precursor subunit requiring two proteolytic cleavages. The first cleavage causes the removal of a signal peptide involved in transporting the protein into the lumen of the endoplasmic reticulum (Tumer et al., 1981). These proglycinin subunits aggregate into trimers in the endoplasmic reticulum, and move to the vacuolar protein bodies (Dickinson et al., 1989). The second cleavage takes place post translationally within the vacuolar protein bodies, resulting in fragmentation of the proglycinin subunit into its constituent 40 kDa acidic and 20 kDa basic polypeptide chains.

The amino acid sequence and the genes that encode the soybean glycinins were characterized by Nielsen et al. (1989). Plietz et al. (1987) used this amino acid sequence of glycinin to compare the sequence homology of the acidic and basic subunits, by computer analysis. Using the results of this analysis, the secondary structure and arrangement of the subunits in the native glycinin were predicted. Based on these investigations, it was shown that there was a high sequence similarity between the N-terminal region of the acidic and basic subunit chains. The two N-terminal regions had similar secondary structure and their sequences were conserved. These structural and sequence similarities indicated that the N-terminal region could have a functional or structural meaning. An alternating sequence of β-strands and β-turns dominated within the N-terminal region of the acidic and basic subunits. The C-terminal region of the acidic chain was strongly hydrophilic in contrast to the hydrophobic C-terminal region of the basic chain. Based on these data, the model proposed by Plietz et al. (1987) was as shown in Figure 1.1.

According to this model, the N-terminal region of the acidic and basic chains

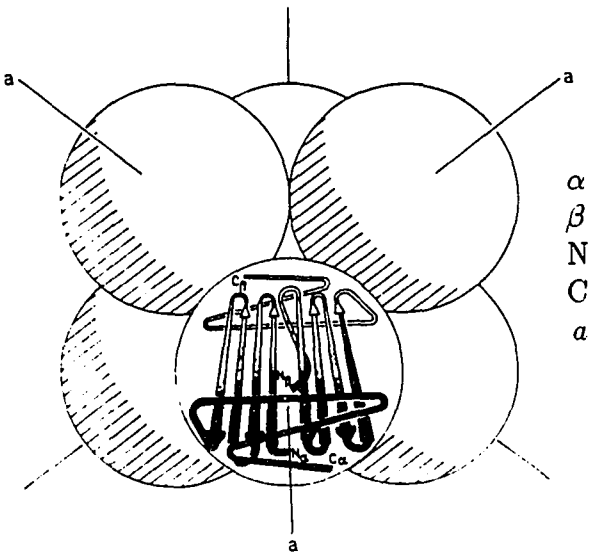


Figure 1.1: Hypothetical model of the arrangement of the acidic chain and basic chain of each subunit within the hexamer molecule of the glycinin (Plietz et al., 1987)

formed the two domains of each globulin subunit, and were arranged at the same distance around the center of the oligomeric molecule. The strongly hydrophilic C-terminal region of the acidic chain was situated at the surface of the protein molecule and it protected the domains from the solvent. The more hydrophobic C-terminal region of the basic chain was situated near the center of the molecule, and it stabilized the oligomeric structure by hydrophobic interaction with the same sequence regions of the other five subunits. Consequently, each subunit had a twofold pseudosymmetry and the glycinin molecule had a threefold pseudosymmetry. This symmetry was confirmed by X-ray crystallography (Plietz et al., 1987; Dickinson et al., 1989). Using Fourier transform infrared spectroscopy, Dev et al. (1988) have shown, that the native

structure of soybean glycinin consisted mainly of β-sheet, β-turns and disordered structure with very little α-helix. Further, their investigations indicated that on denaturation with urea, the β-sheet structure disappeared almost completely. In the native state, the acidic and basic polypeptide chains of each glycinin subunit were linked by a single disulfide bond (Nielsen, 1985). Catsimpoolas (1969) reported that the glycinin molecule had 44 cysteine residues, but according to Rothenbuhler and Kinsella (1986), soy glycinin had only 17 to 20 disulfide bonds per glycinin molecule.

The earlier models proposed for the quaternary structure of glycinin (Catsimpoolas, 1969; Badley et al., 1975) suggested that the molecule consisted of two rings of subunits stacked one on top of the other. There were six subunits per ring, and the rings were composed of alternating acidic and basic subunits, with ionic interactions contributing to the stability of the oligomer. However, using reassembly studies with the acidic and basic chains of glycinin, Dickinson et al. (1990) have demonstrated that the acidic chain was less important for assembly of the molecule than the basic chain. This was not consistent with an "alternating acidic/basic" model, because in such a model one would expect the two domains to be equally important for assembly. Instead, these results supported the model proposed by Plietz et al. (1987); Dickinson et al. (1989); Nielsen et al. (1989); that placed the basic domains at the center of a trimer ring and the acidic domains on the outside. Dickinson et al. (1990) have also shown that the acidic domains of glycinin were more important than the basic domains, for keeping the molecule in solution.

Grinberg et al. (1989) investigated the thermodynamic characteristics of soybean glycinins using differential scanning microcalorimetry. These researchers demonstrated that the enthalpy and entropy of denaturation, decreased monotonically as

the temperature was lowered below denaturation temperature, and became negative at physiological temperature. These regularities caused them to conclude that hydrophobic effects played a substantial role in the stabilization of the folded conformation of glycinin. At 110°C, the calculated specific enthalpy of denaturation for glycinin was 31 ± 4 Jg^{-1}. Consequently, the glycinin globulin was categorized as a protein with noncompact structure. According to Privalov (1979), the denaturation enthalpy of proteins with compact structure was approximately 55 Jg^{-1} at 100°C, while that of proteins with noncompact structure was in the range of 30–38 Jg^{-1} at 110°C.

β-Conglycinin β-Conglycinin is a major soybean protein fraction, composed of several isomeric forms (B_0-B_6), containing three subunits (α, α' and β) in different ratios (Thanh and Shibasaki, 1976). The molecular weights of these subunits are 57 kDa, 58 kDa and 42 kDa for α, α' and β subunits, respectively. The N-terminal amino acids of α, α' and β subunits have been identified as valine, valine and leucine (Hirano et al., 1987). These researchers have also shown that there was internal region sequence homology among all three subunits, but N-terminal region sequence homology existed only between the α and α' subunits. At high ionic strength ($I > 0.5$) or at acidic pH ($pH < 4.8$), the protein existed as a globular protomer conformation (trimeric structure), while at low ionic strength ($I < 0.2$) in the pH region 4.8–11.0, it dimerized into a 9S hexameric structure (Nielsen, 1985). Rapid interconversion between these two structures was observed in the ionic strength region of 0.2–0.5. At very high pH (≥ 12), the polypeptide dissociated irreversibly. However, at lower pH values, the dissociation of subunits was reversible (Thanh and Shibasaki, 1979). β-

Conglycinin was shown to be a glycoprotein with a total carbohydrate content of 5% (Koshiyama, 1968). Each subunit had one or two N-linked glycosyl groups that consisted of Asn(NAcGlc)$_2$(Man)$_{7-9}$ (Nielsen, 1985). Iibuchi and Imahori (1978) determined that electrostatic forces were responsible for the dimerization of β-conglycinin into a hexameric structure. Since a plot of the sedimentation coefficient versus pH was similar to the pH titration curve of a carboxylic group, it was concluded that a carboxylic ion played a major part of the electrostatic interaction. Koshiyama (1968) reported that the β-conglycinin subunits were held together by noncovalent bonds and that tyrosyl -OH groups contributed greatly to these noncovalent bonds. It was demonstrated that the two disulfide bonds present per β-conglycinin molecule were buried in the hydrophobic region of the molecule and thus had no effect on the dimerization of the molecule to its hexameric structure.

Recently, Deshpande and Damodaran (1990) reported (using far UV circular dichroism) that the secondary structure of soy β-conglycinin was characterized predominantly by β-sheet and β-turns, and that of the 15 tyrosyls present per subunit, 10 were exposed to the surface. The surface hydrophobicity of β-conglycinin was determined to be 1565, using *cis*-parinaric acid as a hydrophobic probe. This high value of surface hydrophobicity showed that β-conglycinin had a strongly hydrophobic surface topology, which was much greater than the surface hydrophobicity of other legumin-like proteins with sedimentation coefficient 7S. In contrast, the surface hydrophobicities of phaseolin and vicilin were 51 and 906, where phaseolin and vicilin were the legumin-like proteins of dry beans and field peas, respectively.

Soy lipoxygenase Lipoxygenase (EC 1.13.11.12) catalyzed the hydroperoxidation of certain unsaturated fatty acids and various polyunsaturated lipids containing *cis*,*cis*-1,4-pentadiene moieties. This enzyme initiated oxidation as soon as it was brought in contact with soybean oil, in the process of crushing the dry seeds. The oxidized products further decomposed into medium chain aldehydes and alcohols (due to the action of hydroperoxide lyase) which were major constituents of the undesirable grassy beany flavor in soybean products (Matoba et al., 1985a). When lipoxygenase and hydroperoxide lyase acted on free fatty acids and triglycerides, n-hexanal was reported to be the main product formed (Matoba et al., 1985b). Several favorable functions of the lipoxygenase activity were the formation of intracellular free radicals and fatty acid hydroperoxides, which may be involved in plant senescence (Leshem et al., 1981) and in the synthesis of some regulatory compounds (Kacperska and Kubacka-Zebalska, 1985). The peak activity of the three lipoxygenase isozymes in soybeans (L-1, L-2 and L-3) were at pH 9, pH 6.6 and pH 6.8, respectively, when linoleic acid (18:2) was used as the substrate (Axelrod et al., 1981). Since the pH of the soybean extract was 6.5–7.0 (Matoba et al., 1985a), lipoxygenase isozymes L-2 and L-3 were reported to be more effective at carbonyl production than L-1 (Grosch and Laskawy, 1975).

Zhuang et al. (1991) found that among the lipoxygenase isozymes, the isozyme L-2 was the most effective in producing C_6 aldehydes from linoleic acid (18 : 2). According to this group, the most preferred substrates for lipoxygenase activity were free fatty acids of chain length 18–20 with a *cis*,*cis*-1,4-pentadiene structure. Fresh, high-quality mature soybean seeds generally contained only a few tenths of a percent of free fatty acids. However, lipoxygenase L-2 could also act on triglycerides.

The hydrophobic effect Hydrophobic bonding is a type of bonding that may contribute to the interaction between the ligand and the protein, in a protein–ligand complex. The characteristics of this type of bonding are discussed in the following paragraphs.

Nonpolar groups/molecules such as hydrocarbons are not soluble in very polar solvents like water. Occasionally, nonpolar groups/molecules could be maintained in solution in an aqueous medium, either at very low concentrations or when part of a molecule that was soluble in water due to the presence of other polar groups. Under these conditions, if the nonpolar group is exposed to the aqueous medium, its insolubility results in a hydrophobic effect. Accordingly, the hydrophobic effect is defined as the solubilization of hydrocarbons in water. It could be extended to more complex phenomena that occur in an aqueous solution such as the formation of micelles, surface films and the interaction of proteins with hydrophobic ligands. The cohesive forces between water molecules in pure liquid water are hydrogen bonds. According to Tanford (1973), when a nonpolar molecule is dissolved in water, it is unable to form any strong bonds with the water molecules. As a result, the bonds existing in pure water were distorted causing water molecules to readily form cagelike cavities within which the nonpolar solutes were enclosed. In fact, the attraction of nonpolar groups (such as hydrocarbon chains) for each other played only a minor role in the hydrophobic effect. In this system, the force of attraction between water molecules was the greatest, while the interactions between hydrocarbon molecules and water were weaker and those between hydrocarbon molecules were the weakest (Grant and Higuchi, 1990). This unfavorable arrangement of water molecules which took place when there was contact between water and a nonpolar portion of a molecule, caused

a decrease in disorder and a reduction in entropy. When protein molecules were unfolded, there were many nonpolar side chains exposed to the water, but only a few were exposed in the native state. Hence, the native state of the protein was favored (Tanford, 1962). Similarly, when a nonpolar ligand was equilibriated with a protein, due to the above mentioned reasons, the protein–bound form of the ligand was favored more than the unbound form. The differences in solubilizing a hydrophobic solute and a polar solute in an aqueous medium are seen in the thermodynamic parameters as illustrated in Table 1.1. Using the concentration dependence of limiting excess volumes of solubilizing alcohols in water, Franks (1983) has shown that alcohol (including methanol) behave as hydrophobic solutes, despite the presence of the -OH group.

Table 1.1: Limiting thermodynamic excess functions of hydrophobic and polar solutes in aqueous solution (Franks, 1983)

Thermodynamic parameter	Hydrophobic solutes	Polar solutes
Free energy	+	−
Enthalpy	+ or −	−
Entropy	−, $T\|\Delta S\| > \|\Delta H\|$	−, $T\|\Delta S\| < \|\Delta H\|$
Heat capacity	+	− (~ 0)
Volume	−	− (small)
Expansibility (at low temp.)	−	+
Compressibility	− (small)	− (large for ions)

In a water–soluble protein, about 25–30% of the amino acid side chains are generally very hydrophobic, and 45–50% are typically ionic or contained uncharged hydrophilic side chains (Tanford, 1973). The rest have relatively little preference for

being in or out of the aqueous environment. In the native conformation of such a protein, a substantial fraction of the hydrophobic side chains are typically buried in the interior of the molecule, while a major fraction of the hydrophilic side chains are on the surface of the protein. In a polypeptide chain, the hydrophobic amino acids are not grouped together, but are interspersed with amino acids having ionic or polar side chains, each individual protein polypeptide chain having a unique sequence. As a consequence, the complete removal of hydrophobic side chains from contact with water is generally not possible. In most native proteins, some hydrophobic groups remain exposed at the molecular surface or in crevices. These "hydrophobic patches" may act as binding sites for hydrophobic ligands. As illustrated in Figure 1.2, the hydrophobic parts of the binding sites are circumscribed and limited in the length of hydrocarbon chain that could be accommodated.

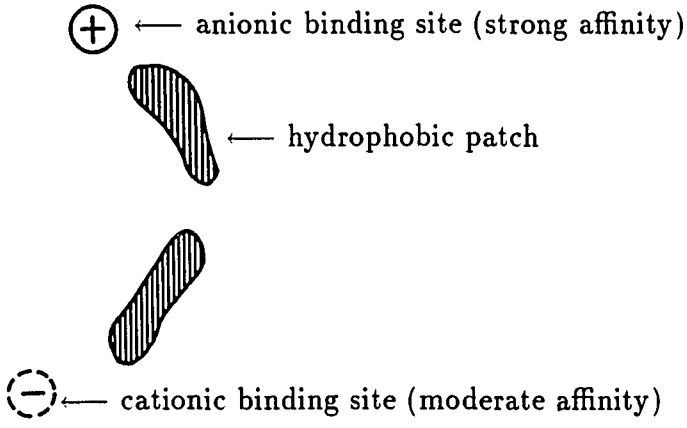


Figure 1.2: Schematic diagram of a binding site of native serum albumin for amphiphiles (Tanford, 1973)

The hydrophobicity of protein molecules could be determined as surface hydrophobicity (S_0) and exposed hydrophobicity (S_e). Both these parameters were useful in correlating with protein functionality. The binding of *cis*-parinaric acid to proteins, in many cases was reported to involve ionic as well as hydrophobic interactions (Mattarella and Richardson, 1983). It was suggested that the alkane binding procedure of Mohammadzadeh-K et al. (1969) might be a better indicator of protein hydrophobicity than the fluorescent probe method using *cis*-parinaric acid. Using whey protein concentrates, the binding of pentane, heptane and nonane were studied (Mangino et al., 1985). As the chain length of the alkane increased, the proportion of alkane bound decreased. It appeared, that as the alkanes increased in size, larger hydrophobic areas were required for binding. With fewer of these regions present in the protein, less alkane was bound. When the whey proteins were denatured using urea and dithiothreitol, the amount of heptane bound increased, indicating exposure of hydrophobic sites buried in the protein molecule.

Flavor problem in soybean products

The off-flavor problem is one of the major problems faced by the soybean industry. Numerous researchers have investigated several aspects of this problem. However, since this problem has not been completely solved, it is important to review the literature published on this subject, in order to understand the complexity of the problem and to lay the basis for the study reported in this dissertation. In this section, the citations have been divided into three categories. First, the investigations on the identification of the flavor compounds responsible have been reviewed. Second, the attempts made to reduce this off-flavor in soy products using physical

methods and using enzymes have been reviewed. Lastly, the investigations on the binding parameters have been reviewed.

Identification of flavor compounds As early as 1969, Mattick and Hand reported that the volatile component responsible for the raw bean odor and flavor in soybean products was ethyl vinyl ketone. They have also observed that this flavor was not present in intact raw whole soybeans, but developed immediately after maceration of the beans. The formation of this product was thought to be due to enzymatic action, as its appearance was rapid and present only in unblanched soybean products.

Hsieh et al. (1981) carried out an extensive study of the volatile components of defatted soy flour. The volatiles were distilled off, frozen in cold traps, and analyzed by gas chromatography (GC), mass spectrometry and infrared spectroscopy. Twenty five compounds were identified, including nine alcohols, six aldehydes, nine ketones and 2-pentyl furan. Among the identified compounds, 2-pentyl furan and ethyl vinyl ketone were the key compounds. All the compounds identified in this study were postulated as autoxidative decomposition products of soy lipids. Thus, the presence of these compounds in soy flour was attributed to the incomplete removal of lipids in soy flour.

In a review article published by Maga (1973) on the flavor aspects of soy products, the main reason preventing the widespread utilization of soy products in American and European food patterns has been identified as the characteristic soybean flavor. Even though several compounds contributed to the volatiles of soy products, due to its low flavor threshold, hexanal was one of the main components giving rise to the green bean-like flavor in raw soybeans. It has been pointed out, that although

hexane was efficient in removing the oil portion of soybeans, hexane usually did not remove the characteristic bitter beany flavor normally associated with soybeans. A patented method using equal volumes of ethanol, ethyl acetate and acetone to extract the fat, taste and odor compounds from soybeans was also mentioned in this article.

In a review by Cowan et al. (1973), lipoxygenase and linoleic acid were recognized as the primary sources responsible for the soy product flavor, when soybeans were partially processed before destroying enzymatic activity. In an investigation of lipid-derived flavors of legume protein products, the major contributors to the green beaniness of soybeans were identified as 3-*cis*-hexenal, 2-pentyl furan and ethyl vinyl ketone (Sessa and Rackis, 1977). A new approach involving infusion of antioxidants into the intact seed to control lipid deterioration during processing and storage was proposed to minimize flavor formation, without subsequent undesirable changes in protein. The antioxidants suggested for use were nordihydroguariaretic acid, propyl-gallate and α-tocopherol and the method of infusion of these chemicals into dry seed was by the aid of solvents such as dichloromethane and acetone.

Reduction of soy off-flavor A chicken soup was formulated with added textured soy protein (Griffith Laboratories, Scarborough, Ontario), hoping to mask the soy off-flavor with that of chicken (Malcolmson et al., 1987). At very low levels of soy protein, even though the soy off-flavor was not detectable, there was a reduction in the perceived chicken flavor. An unpleasant soy flavor was detectable when the level of soy was increased to 16%. Even with an increased chicken flavor, the soy flavor could not be masked at this level. A fermented soymilk beverage was made by Wang et al. (1974) in order to eliminate the off-flavor in soymilk. The beany flavor seemed

to be masked in this milk, which was fermented using *Lactobacillus acidophilus* and *L. bulgaricus*. Crail (1988) used a soy isolate (Edi-Pro A) to replace some meat in a summer sausage formulation, hoping to substitute more expensive meat with cheaper soy proteins without a loss in quality and acceptability. In this study, 2% replacement of meat with soy corresponded to 12.5% total protein replacement. Replacement levels between 0–16% were made, and the consumer preference and texture were evaluated. Although panelists were not able to detect any soy flavor at a level of 2% soy isolate, an off-flavor was detectable at 4% level of soy isolate. There was no difference in texture up to a level of 8% soy isolate. However, at a level of 6% soy isolate, the panelists disliked the product, and totally rejected it at a level of 8%.

Kitamura (1984) screened a large number of samples of soybean seeds, and isolated single and double mutant varieties that lacked the ability to produce either one or two lipoxygenase isozymes. These low lipoxygenase seed types were propagated in greenhouses to produce seeds. However, they have not been developed into successful crop varieties. Although mutant seeds that lacked one or two isozymes had reduced lipoxygenase activity, the beany, grassy off-flavor still developed when they were crushed.

The most effective method of reducing lipoxygenase activity has been either wet or dry heat treatment (Wolf, 1975; Hildebrand and Kito, 1984). Both these methods caused a reduction in protein solubility, while dry heat also imparted a toasted flavor and darkening of color. According to Wolf (1975), using hexane–alcohol to extract the remaining lipid from soybean meal before processing to produce soy isolate, greatly reduced the off-flavor in soy isolate. Borhan and Snyder (1979) were able to reduce the lipoxygenase activity while maintaining a high nitrogen solubility index (NSI)

in whole soybeans, by soaking the beans at 50°C in 15% ethanol for 6 hr. Ashraf and Snyder (1981) have documented that there were still detectable quantities of lipoxygenase activity in soybeans treated according to the method of Borhan and Snyder (1979). An improvement to the above mentioned method as suggested by Ashraf and Snyder (1981) required soaking soybeans at 60°C in 15% ethanol for 5 hr to produce a soymilk free of painty flavor and undetectable lipoxygenase activity.

Arai et al. (1970a) identified n-hexanal and n-pentanol as products of lipoxygenase activity on whole soybean extracts, and proposed a scheme to account for the conversion of *cis*,*cis*-linoleic acid (or its ester) to n-hexanal and/or n-pentanol. Using vacuum distillation, to remove n-hexanal and n-hexanol from an aqueous mixture of soy protein, Arai et al. (1970b) have demonstrated that a small portion of these flavor compounds was resistant to removal. Their findings showed that enzymatic proteolysis destroyed active hydrophobic zones decreasing the binding strength and enabling the release of n-hexanal and n-hexanol. Moreover, the resistance to the release of flavor compounds was greater with denatured soy proteins than with native soy proteins. Thus, they speculated that since these equilibria were in aqueous phase, the participation of hydrogen bonding in the interaction was low and that most of the binding could be attributed to hydrophobic binding.

According to Chiba et al. (1979a), aldehyde dehydrogenase (EC 1.2.1.3) purified from bovine liver mitochondria, together with NAD^+, was successful in removing the green beany flavor in soy protein isolate solutions. This enzyme catalyzed the irreversible conversion of hexanal to hexanoic acid. The beany flavor did not develop in soy protein isolates that were treated with aldehyde dehydrogenase, even after storage for three weeks. However, the authors suggested that it would be impractical

and uneconomical to use this method without devising a way to use the enzyme and NAD^+ efficiently, such as by immobilizing on a solid support. Although bovine liver and yeast aldehyde dehydrogenase, oxidized the soybean protein-bound aldehyde, horse liver alcohol dehydrogenase did not reduce the protein-bound aldehyde (Chiba et al., 1979b). When aldehyde dehydrogenase oxidized aldehydes bound to the soy protein, the resulting carboxylic acid was released from the protein. The affinity of n-hexanal, for aldehyde dehydrogenase from bovine liver and from yeast and alcohol dehydrogenase from horse liver were $K_m = 0.075\ \mu M$, 6 μM and 74 μM, respectively.

Alcohol dehydrogenase (EC 1.1.1.1.) in the presence of NAD^+ has been used to reduce aldehydes to the corresponding alcohols (Eriksson, 1968). Experimentally, the odor threshold value in water was found to be 4.5 ppb for n-hexanal (Guadagni et al., 1963) compared with that of 500 ppb for 1-hexanol (Flath et al., 1967). The odor detectability of aldehydes and alcohols among human subjects was studied by Eriksson et al. (1976). The threshold levels for these compounds were shown to be dependent on the panelists, with a large variation in the range.

The use of bovine liver mitochondrial aldehyde dehydrogenase and bovine liver aldehyde oxidase (EC 1.2.3.1) to oxidize medium chain length aldehydes in soy products were investigated by Takahashi et al. (1979ab). Neither of these enzymes was very effective in oxidizing protein-bound aldehydes. Their substrate specificity was broad and the affinity for n-hexanal was low. Reaction with aldehyde oxidase had to be carried out at pH 9.3 in the presence of ammonium ions, dithiothreitol and FAD.

The aldehyde dehydrogenase oxidation of hexanal in soymilk has been improved by using diaphorase (EC 1.6.4.3), in the presence of NAD^+ (1 μM) (Takahashi et al., 1980). The diaphorase regenerated the NAD^+ consumed in the oxidation of the

aldehyde, and the procedure was successful in reducing the green beany flavor of soymilk.

The involvement of lipoxygenase enzyme in the development of off-flavor in soy products were tested, by comparing soybean lines nearly isogenic to the cultivar Century, that lacked the lipoxygenase isozyme or isozyme combinations L_1, L_2, L_3, $L_1 + L_3$ or $L_2 + L_3$ (Davies et al., 1987). Near isogenic lines were used to ensure that any effects observed were due to the elimination of lipoxygenase isozymes, and were not due to other unrecognized genetic differences between lines. Soy flour and soymilk preparations were subjected to sensory evaluations, to test for dairy, cereal, beany, rancid, oily, chalky, bitter and astringent flavors. The L_2–less line showed the greatest difference in score, and it was for the rancid flavor. The data indicated that genetic elimination of the L_2 isozyme may reduce some of the off-flavors associated with soy products. However, the commercial feasibility of the removal of the L_2 isozyme has not been evaluated.

Parameters involved in soy off-flavor binding Beyeler and Solms (1974) investigated the binding of several flavor compounds to soy proteins, using an equilibrium dialysis method. Their investigations revealed that the overall binding constants K ranged from 0 to 10^4 M^{-1}, increasing in the order of phenylalanine, aniline, dimethylpyrazine, carboxylic acids, alcohols, ketones and aldehydes. Phenylalanine exhibited no binding affinity. The interactions were characterized by weak and unspecific binding forces and almost unlimited binding capacity. They speculated that both electrostatic and hydrophobic bonding were involved in this interaction. The ligand concentrations used were from 10–80,000 ppm and the molecular weight of the

soy protein was taken to be 50 kDa. Their data indicated that when the pH changed from 4.5 to 7, the binding constant increased at constant temperature, as shown in Table 1.2.

Table 1.2: Overall binding constant (K) of several ligands with soy protein isolate at 20°C and two different pH values (Beyeler and Solms, 1974)

Ligand	$K \times 10^{-3}(M^{-1})$	
	pH 4.5	pH 7.0
2-butanone	4.975	5.174
1-butanol	2.100	2.841
phenol	3.159	4.232
vanillin (p-aminobenzoic acid)	2.040	2.200

Solms et al. (1973) reported that in the native structure of proteins, the hydrophobic side chains of amino acids were invariably on the inside of the spherical protein and that the polar groups were on the outside. It was also mentioned that soy proteins had compactly folded structures, including interior hydrophobic regions. According to these researchers, the flavor binding mechanism was hydrophobic, which occurred with the unfolding and destruction of the tertiary structure, liberating hydrophobic binding zones from the interior of the protein molecule.

The interaction of several flavor compounds with soy proteins, in an aqueous model system was studied by Gremli (1974). During this study, equilibrium was attained in 30 min. It was shown that alcohols did not bind to soy proteins, but that aldehydes and ketones did, with the unsaturated aldehydes having the strongest binding. Using a vacuum distillation technique, the author concluded that a small

percentage of the aldehydes were permanently bound, due to an irreversible reaction with the protein, and that a greater portion was bound reversibly.

A labeled ^{14}C-benzyl alcohol was used by King and Solms (1979) to investigate the binding of flavor compounds to a denatured soy protein product (Soyamin 90, Lucas Meyer Co., Denmark). The ligand was equilibriated with the protein, and the suspensions were centrifuged (10 min at 200×g). The ligand in the supernatant was considered as the free ligand, and the ligand in the pellet was considered as the bound ligand. They were able to show that a mixture of dissolved protein and lipid (coffee whitener) was able to decrease the amount of flavor compound absorbed by the Soyamin 90 in the medium, as compared to a control mixture of Soyamin 90 without coffee whitener.

The binding of 2-heptanone, 2-nonanone and nonanal to bovine serum albumin in an aqueous model system, has been studied by Damodaran and Kinsella (1980). Their investigations have shown that the enthalpy of binding was independent of temperature and that the binding was entropy driven, which caused them to conclude that the binding was hydrophobic in nature. The possibility of nonanal forming Schiff bonds with the ϵ-amino groups of lysine was ruled out, by showing that there was no reduction of the available ϵ-amino groups upon formation of the protein–nonanal complex.

Damodaran and Kinsella (1981a), investigated the thermodynamic effects of the binding of 2-heptanone, 2-octanone, 2-nonanone, 5-nonanone and nonanal to soy proteins. An equilibrium dialysis method was employed, with the ligand content in the two compartments analyzed by isooctane extraction, followed by GC analysis. The binding affinities for 2-heptanone, 2-octanone, 2-nonanone, 5-nonanone and nonanal

to native soy protein were 110, 310, 930, 541 and 1094 M^{-1} K_{eq}, respectively. These results indicated a decrease in hydrophobic interaction, caused by a decrease in chain length of the hydrophobic group in the ligand. However, they also reported that the binding affinity of 2-nonanone to soy protein at 5°C was 2000 M^{-1} while at 25°C and 45°C it was 930 M^{-1}. They speculated that this was caused by a structural rearrangement of the subunits in soy protein, from a more ordered state to a less ordered state at lower temperatures. Even though the binding affinity was not independent of temperature, their final conclusion was that the binding was hydrophobic in nature. In a companion study, they investigated the affinity of glycinin and β-conglycinin to 2-nonanone separately (Damodaran and Kinsella, 1981b), using the same method of equilibrium dialysis. The binding affinity of β-conglycinin was the same as that of whole soy protein, while the glycinin fraction exhibited almost no affinity for 2-nonanone. Since glycinin had 21 disulfide bonds per molecule and β-conglycinin had none, they speculated that the network of disulfide linkages in glycinin may have rendered the hydrophobic regions inaccessible to the ligand. They postulated that in the glycinin molecule, the cavity at the center of the double doughnut structure proposed by Badley et al. (1975) was the site of hydrophobic interaction. In the presence of urea, structural changes in soy proteins were observed by using changes in the fluorescence behavior. Although the nature of binding was concluded to be hydrophobic, in the presence of urea, a decrease in the binding affinity of soy proteins to 2-nonanone was observed. Thus, they concluded that by reversibly altering the quaternary structures, it may be possible to remove the bound off-flavor from these proteins. The method suggested for this, was treating soy protein or soy flour with 1–2 M urea followed by dialysis, to remove urea as well as previously bound

carbonyls.

Aspelund and Wilson (1983) studied the thermodynamics of the off–flavor interaction with soy protein, in a dry system. A soy protein isolate (Edi-Pro A, Ralston Purina Co., St. Louis, MO) was used as the packing material in a GC column, and this column was set up in a GC. Samples (5 μL) of the flavor compound volatiles were injected onto this column, and the retention time was determined. These analyses were performed at three different temperatures, and the thermodynamic parameters were calculated. The Gibbs free energy decreased with increasing temperature, indicating a physical adsorption process which was less favorable at high temperatures. Their observations revealed that alcohol had the strongest adsorption, while hydrocarbons had the weakest. Aldehydes, ketones and methyl esters had similar adsorption characteristics. The authors concluded that the binding reaction was being driven by the enthalpy of adsorption in gaseous systems. Hydrocarbons interacted with soy proteins by nonspecific van der Waals forces, while alcohols interacted with two hydrogen bonds and van der Waals forces. Aldehydes, ketones and esters interacted with one hydrogen bond and van der Waals forces.

The binding of n-hexane, 2-pentanone, 2-hexanone, 2-heptanone, n-hexanol and 1-pentanol to a soy protein isolate (Edi-Pro A) was studied by Crowther et al. (1981). The protein isolate was subjected to heat by autoclaving and/or shear by extrusion. The moisture content was controlled (29% and 40%), and two temperatures (100° and 121°C) were used. The protein was packed into a glass GC column. The headspace volatiles of the ligands being studied were injected into the column, and the retention time was analyzed. Autoclaving greatly reduced adsorption (47–49% for ketones, 35–40% for alcohols and 43% for hexanal) while shearing had a significant effect only on

the alcohols (increase of 14–46%). In this dry system, the extent of binding was the reverse of the order found in an aqueous solution of soy proteins. The alcohols had the highest affinity and the carbonyls followed. It was concluded that the mechanism of binding involved van der Waals forces and hydrogen bonding.

The binding of hexanal to purified glycinin and β-conglycinin was studied in an aqueous model system (O'Keefe et al., 1991b). The number of sites for the binding of hexanal with glycinin and β-conglycinin in tris buffer were 96 and 32, and the affinity constants were 270 and $256 M^{-1}$, respectively. The effect of NaCl, NaN_3 and β-mercaptoethanol on the binding, the change in hydrophobicity of the proteins and the change in the availability of lysine upon ligand binding, were determined. In this study, the turbidity of the protein solution increased noticeably, when the concentration of hexanal was above 300 ppm. The effect of temperature on the binding was also investigated by O'Keefe et al. (1991a). The temperature did not affect the number of binding sites. The number of sites for hexane was reported to be higher than that for the other ligands with β-conglycinin, but lower with glycinin. The number of sites was lower for hexanol compared to hexanal and 2-hexanone, for both soy proteins. With straight chain aldehyde ligands, the binding constants increased as chain length increased. Since the quantity $T\Delta S$ was greater than ΔH, it was concluded that the binding process was entropy driven.

Analysis of ligand binding to protein

Sebille et al. (1990) have reviewed the separation procedures used to study ligand binding to protein. According to this review, the procedures used fall into four main categories, which are equilibrium dialysis, ultrafiltration, liquid chromatogra-

phy (with the ligand and protein in the mobile phase) and affinity chromatography (with either the ligand or the protein immobilized). The ligand that was to be used in the present study was an aldehyde that was known to react with the materials used to make the equilibrium dialysis and ultrafiltration apparatus. Further, there were no known chromatographic media that had either the protein or the ligand of interest, immobilized. Hence, the only method feasible seemed to be based on liquid chromatography with the ligand and the protein in the mobile phase.

Several researchers have used the Hummel and Dreyer (1962) method and several modifications of it to study ligand–protein binding (Colman, 1972; Acred et al., 1963; Brumbaugh and Ackers, 1971; Thuaud et al., 1983). The Hummel and Dreyer method is a simple procedure, analogous in principle to equilibrium dialysis, devised to detect reversible interactions between macromolecules (proteins) and substances of low molecular weight (ligands). In this method, the protein is equilibriated with the ligand and eluted on a size exclusion column, using an eluent that has the ligand incorporated into it, at the same concentration as in the binding assay mixture. The choice of the gel for the size exclusion column is such that it excludes the protein, while allowing the ligand to traverse through it. The concentration of the ligand and the protein are monitored at the column outlet, using suitable analysis techniques. At the time the protein elutes from the column, there is an increase of the ligand concentration (above the equilibrium level) eluting from the column, while there is a trough in the ligand concentration (below the base line level) soon after the protein peak emerges. The amount of free ligand removed from the eluent solution as displayed by the trough, is equal to the excess concentration of the ligand found in the protein peak. The free ligand concentration is the ligand concentration in the

eluent, while the amount bound is the amount of ligand in the protein peak or the trough. Thus, these values could be used to study the binding of this ligand to the protein used. Fairclough and Fruton (1966) used a Sephadex G-25 column of 48″ bed length to achieve complete equilibrium between the ligand–protein complex and the mobile phase. In the present study, a long column (90 cm) was used to investigate the presence of tightly bound ligands, by achieving complete equilibrium between the ^{14}C-heptanal–protein complex and the eluent.

Tightly bound versus loosely bound ligands Tightly bound, loosely bound, reversibly bound and irreversibly bound are all terms used in describing ligand binding to proteins. These are all relative terms and there are no clear cut demarcations between the pairs. Hence, the definitions and usage of these terms during previous investigations are discussed below. The fact that a ligand which is tightly bound to a protein under certain conditions, may not necessarily be tightly bound under a different set of conditions, is also discussed.

Cantley and Hammes (1975) used a method of eluting a ligand–protein complex two times through a Sephadex G-25-medium column (1 cm i.d.×50 cm) to distinguish the "exchangeable" ligands from the "tightly bound" ligands. If the complex dissociated during elution, it was defined as an "exchangeable" ligand, while an undissociated ligand was defined as "tightly bound".

Chloroplast membranes, like other energy transducing membranes had a coupling ATPase with especially tight binding sites for adenine nucleotides (Harris and Slater, 1975). Membranes washed several times with 10 mM sodium pyrophosphate still contained 2.5 nmol ATP and 1.3 nmol ADP bound per mg of chlorophyll. When the

ATPase nucleotide complex was illuminated in the presence of labeled nucleotides [^{3}H]ATP or [^{3}H]ADP and a redox mediator, practically all the tightly bound nucleotides were exchanged for labeled nucleotides. This experiment demonstrated that it was possible for the tightly bound ligands to be released under a different condition, such as energization of the membrane.

The binding of long chain free fatty acids to bovine serum albumin was studied by Spector et al. (1969). The model consistent with their data had six high energy binding sites and a large number of weak binding sites. The apparent association constants at 37°C and pH 7.4 were 10^6 M^{-1} to 10^5 M^{-1} for the high energy sites, while that for the weak sites were 10^3 M^{-1}.

Berger et al. (1989) studied the binding of ADP and ATP to spinach coupling factor CF1, and determined that there were six binding sites, out of which three were of high affinity and the other three were of low affinity. The dissociation constants for the low affinity sites were 15–20 10^{-6} M, while those for two of the high affinity sites were 1–5 10^{-6} M. The binding to one of the high affinity sites was determined to be not entirely reversible. A modification of the Hummel and Dreyer method was used in this study. The authors showed that for a hypothetical case of a macromolecule–ligand complex, which obeyed first order kinetics during dissociation, and associated at the rate of 10^6 $M^{-1}s^{-1}$, the loss of binding during separation reached ten percent in 0.1 s for a dissociation constant $K_D = 10^{-6}$ M or in 1 s for $K_D = 10^{-7}$ M. It was shown that the factor that determined whether a protein–ligand complex could survive zonal gel filtration undissociated, was not its dissociation constant, but the ratio (K_{off}) dissociation constant : association constant (Nimmo and Bauermeister, 1978). When ($K_{off} \geq 0.25$ μM, no protein bound ligand was detectable upon elution

through a 200 mL column of Sephadex G-75.

Loo et al. (1984) established that prednisolone, was a synthetic corticoid that bound to both corticosteroid binding globulin and albumin in human serum. The binding affinity of the steroid for the globulin was higher ($K = 3 \times 10^7$ ml^{-1}) while that for the albumin was lower ($K = 2 \times 10^3$ ml^{-1}). They described a high performance liquid chromatographic gel permeation procedure, which allowed prednisolone bound to albumin to completely dissociate during chromatography, without affecting the binding of the steroid to high affinity proteins. The tightly bound steroid–globulin complex was not dissociated during the chromatographic procedure.

Modification of proteins

In order to achieve the objective of this project, the effect of changing the protein surface on the binding of aldehyde was studied, by modifying the electron rich amino acids. It was hoped that this would enable the identification of the types of bonds involved in the interaction between aldehydes and soy proteins.

Amino groups The free amino groups of proteins could be converted to the N,N-dimethyl derivatives by reaction with formaldehyde and a reducing agent. Although $NaBH_4$ had been used as the reducing agent for this purpose in the past, it also reduced aldehydes, ketones and disulfide bonds. Jentoft and Dearborn (1979) used a milder reducing agent $NaCNBH_3$ which permitted the reaction to be carried out at neutral pH (reduction by $NaBH_4$ was efficient at pH=9) which was less destructive to the protein. $NaCNBH_3$ being a more selective reducing agent, did not reduce the aldehydes and ketones, but readily reduced the Schiff bases. Formalde-

hyde, together with $NaCNBH_3$ methylated only the ϵ-amino groups of lysine residues and the α-NH_2 terminus by reducing the Schiff bases. However, it did not reduce the aldehyde or the disulfide bonds. The amino acid residues arginine, histidine, methionine, tryptophan, tyrosine, serine, asparagine and cysteine were not methylated by this procedure. Cabacungan et al. (1982) and Dufour and Haertlé (1990) used alkyl boranes to reductively alkylate the lysine residues of proteins. Fretheim et al. (1979) were successful in alkylating the amino groups of lysine residues with carbonyl reagents in the presence of sodium borohydride. With smaller carbonyls such as butanal, disubstitution was also obtainable, while larger carbonyls as benzaldehyde gave only monosubstituents.

Tyrosyl groups In 1963, Shaltiel and Patchornik used acetic anhydride to O-acetylate the phenol groups of the tyrosyl residues. The difference in molar extinction coefficients of the O-acetyl tyrosine and the tyrosine, under the same conditions were used to analyze the degree of acetylation. At pH 7, the molar extinction coefficient at 275 mμ for O-acetyl tyrosine was 120 and that of tyrosine under the same conditions was 1400.

N-acetyl imidazole was used by Simpson et al. (1963) for the O-acetylation of tyrosine as it was a milder reagent than acetic anhydride. According to these researchers, acetic anhydride unfolded the protein while N-acetyl imidazole did not denature the protein. Riordan et al. (1965) used N-acetyl imidazole for O-acetylation in preference to acetic anhydride, since acetic anhydride was reported to acetylate ϵ-amino groups as well. Their investigations revealed that N-acetyl imidazole acetylated only the exposed tyrosyl residues in native proteins, while it acetylated all the

tyrosyl residues in denatured proteins. A similar method of O-acetylation was used by Kronman et al. (1971) and Burstein and Patchornik (1972).

Sulfhydryl groups Cole et al. (1958) reacted human hemoglobin with iodo acetic acid to convert cysteine to S-carboxymethylcysteine at pH 9. In this study, denaturation with lauryl sulfate was used to ensure that all the SH groups were exposed to the reagent. Crestfield et al. (1963) also used 2-iodoacetic acid to S-carboxymethylate sulfhydryl groups at pH 6.0–8.5. The reaction was stopped by eluting the reaction mixture on a Sephadex G-75 size exclusion column. Sodium tetrathionate was used by Rothenbuhler and Kinsella (1986) to S-sulfonate the free SH groups in proteins.

Synthesis of aldehydes

The synthesis of a ^{14}C-labeled aldehyde was necessary to carry out the investigations reported in this dissertation. The pertinent literature is reviewed in the following paragraphs. A summary of the methods available for synthesizing the major functional groups in organic compounds was published by Larock (1989). The criteria used in selecting the methods listed in this publication were, the generality in scope, the availability of reagents and the resulting yield (at least 50%). A survey of the methods available for the synthesis of a ^{14}C-labeled aldehyde revealed that due to the restrictions imposed by the availability of ^{14}C-labeled reagents/substrates, only three synthetic routes were feasible. They were based on the use of the Grignard reagent, the oxidation of an alcohol and the reductive hydrolysis of a nitrile to the aldehyde.

Bachman (1943), used the Grignard reagent formed from n-amyl bromide and ethyl orthoformate, to synthesize hexaldehyde. The reaction was performed in a dry ether medium, the acetal formed was hydrolyzed by sulfuric acid and the liberated hexaldehyde was distilled off.

Dipyridine-chromium(VI) oxide was used by Collins et al. (1968) to oxidize alcohols to aldehydes. When using a 6 :1 mole ratio of complex : alcohol at 25°C, the reaction was complete in 15 min. A 93% yield of heptanal from 1-heptanol was obtained. Corey and Suggs (1975) improved on the method of Collins et al., by using pyridinium chlorochromate as the oxidizing agent. In this method, a 1.5:1 mole ratio of complex : alcohol was used, and the resulting yield was 78%.

Alkyl nitriles were synthesized by reacting primary or secondary alkyl halides with sodium or potassium cyanide in a dimethyl sulfoxide (DMSO) medium (Friedman and Shechter, 1960). In this method the yield of nitrile was high with primary alkyl halides and short chain alkyl halides. Sodium cyanide was a better reagent for nucleophilic substitution than potassium cyanide. The resulting nitrile was isolated by diluting with water followed by diethyl ether extraction and distillation.

The conversion of a nitrile through imino-chloride into an aldehyde with the same number of carbon atoms, was documented by Stephen (1925). The reducing agent used was anhydrous stannous chloride dissolved in ether, saturated with hydrogen chloride. The nitrile was added to this reducing agent, the resulting imino-chloride was hydrolyzed by warm water, and the aldehyde was extracted and distilled off.

Nitriles were converted to aldehydes by the partial reduction by lithium triethoxyaluminohydride, followed by hydrolysis (Brown and Garg, 1964). The reagent lithium triethoxyaluminohydride was prepared in situ by reacting lithium aluminum

hydride with ethanol or ethyl acetate. A variety of nitrile substrates from n-butyronitrile to *p*-anisonitrile were used.

Winterfeldt (1975) reviewed the use of diisobutyl aluminum hydride (DIBAL-H) and triisobutyl aluminum (TIBA) as reducing agents in organic synthesis. The advantages of using DIBAL-H or TIBA over the use of lithium aluminum hydride, based on the milder reaction conditions required and the selectiveness of reduction, were discussed.

Several authors have reported DIBAL-H as a very selective reducing agent in organic synthesis. The reduction was carried out by Marshall et al. (1970), at $-60°C$, using saturated NH_4Cl to stop the reaction. A solution of 5% aqueous sulfuric acid was used as the hydrolyzing agent, and the aldehyde was isolated by ether–hexane extraction. Bradsher and Edgar (1981) used the same method as that of Marshall et al. (1970), but the reaction was performed at $-72°C$, and ether was used to extract the resulting aldehyde from the reaction mixture. In this method, sodium chloride was used to enhance phase separation in the work up of the reaction mixture. A similar method reported by Cohen et al. (1976) used a reaction temperature of $-70°C$ while ethyl acetate and aqueous NH_4Cl were used to stop the reaction. The resulting imino complex was hydrolyzed with 1N aqueous H_2SO_4. Taber et al. (1987) carried out the DIBAL-H reduction of nitrile in a tetrahydro furan (THF) medium at $0°C$. The imine formed was decomposed by saturated NH_4Cl, followed by 10% aqueous H_2SO_4. Baasov and Sheves (1985) used DIBAL-H to reduce a cyanoester at $-78°C$, while using ethyl acetate to stop the reaction. Overman and Burk (1984) initiated the DIBAL-H reduction reaction at $-70°C$. After 2 hr, the reaction mixture was warmed to $0°C$, and NaF was used to decompose the imino complex formed.

Statement of the Problem

Soy proteins have excellent functional properties that lend themselves to improving the quality of processed foods and in substituting for more expensive meat proteins. Off-flavor problems inhibit their extensive use in human foods, by making them unacceptable to the consumers (Maga, 1973). Medium chain aliphatic aldehydes that bind to soy proteins have been identified as the main source of this off-flavor (Cowan et al., 1973).

As described in the review of previous work, many researchers have attempted to determine which compounds caused this off-flavor problem, what these compounds bound to, how many of them bound to and how tightly they were bound. All previous researchers have assumed that this off-flavor binding to soy protein was completely reversible. Flavor binding studies under equilibrium conditions have been used to quantitate the reversible binding. Traditional models for the analysis of binding such as Scatchard plots that are based on the assumption of reversibility of binding, were used to analyze and interpret the data.

None of the researchers has analyzed the off-flavor causing compounds in their protein-bound state. The amount of ligand bound to the protein was calculated in one of several methods in aqueous systems. One approach used the difference in the total amount of ligand used and the amount of free ligand remaining (O'Keefe et al., 1991ab). In this method, the amount of free ligand was determined by GC analysis of the headspace. Another method involved liquid extraction of the ligand from the two compartments of the equilibrium dialysis apparatus, followed by GC analysis of the extracted ligand (the protein was present only in one compartment) (Damodaran and Kinsella, 1981a). A third method involved the use of an enzyme

(aldehyde dehydrogenase) and NAD^+ to oxidize the aldehydes (Chiba et al., 1979a). The degree of utilization of NAD^+ was used to quantitate the aldehyde present. Since no more aldehyde could be detected in the system, it was inferred that all the aldehyde was removed by the enzyme. The method reported by Crowther et al. (1981) and Aspelund and Wilson (1983) involved the injection of the off–flavor compound onto a GC column packed with the protein of interest and measuring the retention time of the off–flavor compound for dry system interactions.

However, irreversible flavor binding is probably important due to its ability to alter the flavor profile in a food product. Flavors bound irreversibly could cause a loss of desirable flavors and these off–flavors could be released under different conditions. Direct measurement of the amount of aldehyde bound to the protein is essential, to examine the reversibility of flavor binding to soy protein and to study the mechanism of binding. The use of radiolabeled aldehyde flavor compounds would enable these analyses to be performed at very low concentrations, simulating conditions present in soy foods. Thus, the synthesis of a radiolabeled medium chain aldehyde flavor compound was necessitated.

A careful survey regarding the availability of these radiolabeled flavor compounds at chemical suppliers, revealed negative results. An expensive alternative was to have the aldehyde custom–made by chemical manufacturers. Thus, it was decided to synthesize the aldehyde ^{14}C-heptanal in our laboratory. The method of synthesizing this aldehyde is reported in Chapter 2. The choice of the method of synthesis was restricted by the availability of suitable radiolabeled reagents.

The synthesized ^{14}C-heptanal was used to study the binding of this aldehyde to soy proteins glycinin and β-conglycinin. This study is reported in Chapter 3.

CHAPTER 2. SYNTHESIS OF ^{14}C-LABELED HEPTANAL

Introduction

In this study the objective was to synthesize ^{14}C-labeled heptanal with the highest level of purity possible. During the preliminary stages of this study, several methods of synthesizing ^{14}C-heptanal were attempted unsuccessfully. Finally, the method that yielded good results was based on nucleophilic displacement of 1-iodohexane by $Na^{14}CN$, followed by selective reduction of the nitrile formed by DIBAL-H.

Materials and Methods

Analytical grade reagents were obtained from commercial sources and used as received. Dimethyl sulfoxide (DMSO), ^{12}C-NaCN, H_2SO_4, diethyl ether, CH_2Cl_2, NaF, Na_2SO_4, ScintiVerse (liquid scintillation cocktail), Omni vials (polypropylene scintillation vials) and Chromerge were from Fisher Scientific Co. (Fair Lawn, NJ). Diisobutyl aluminum hydride (DIBAL-H) in CH_2Cl_2 and n-$C_6H_{13}I$ were from Aldrich Chemical Co. (Milwaukee, WI). ^{14}C-NaCN was from Sigma Chemical Co. (St. Louis, MO). The DMSO and CH_2Cl_2 used as solvents were always obtained from freshly opened supplies, in order to prevent any moisture entering the reaction mixture. The glassware was washed in a chromic acid bath (Chromerge and H_2SO_4), rinsed in distilled water, oven dried, cooled as well as stored in dessicators.

Heptanitrile synthesis

The method of synthesizing heptanitrile from iodohexane was adapted from Friedman and Shechter (1960), with several modifications. 1-Iodohexane (4.5g) was added in 30 min, to a rapidly stirred partially–soluble mixture of sodium cyanide (1.35g, 250 μC_i ^{14}C) in DMSO (20 mL) maintained at 60°C. Then, the temperature

$$C_6H_{13}I + NaCN \longrightarrow C_6H_{13}CN + NaI$$

was raised to 90°C and maintained for 15 min. During this period the mixture became more soluble. The reaction was stopped by adding chilled water (25 mL) at 4°C to the mixture. The mixture was cooled and extracted with diethyl ether (4×15 mL), and the combined ether extracts were washed with chilled water (15 mL) at 4°C and dried over anhydrous Na_2SO_4 (×2). The product was distilled through a fractionation column (ACE Glass Inc., Cat. No. 9326) at the rate of one drop/min, analyzed by GC (Varian 3400), and further identified by ^1H-NMR.

GC analysis GC analysis was used to analyze effectiveness of synthesis and the purity of synthesized products, at various stages of the synthesis of ^{14}C-heptanal. A Varian 3400 gas chromatograph (Sugar Land, TX) fitted with a Nukol fused silica capillary column (Supelco Inc., Bellefonte, PA) length 30 m, id 0.32 mm, film thickness 0.25 μm was used. The carrier gas was hydrogen at a flow rate of 4 mL/min, while the makeup gas was nitrogen at a flow rate of 26 mL/min. A flame ionization detector was maintained with an air flow of 300 mL/min and hydrogen flow of 30 mL/min. The injection port and detector were maintained at 210°C and 250°C, respectively. The chromatographic run was started at a column temperature of 40°C,

after 4 min the temperature was programmed up to 160°C in 4 min and was maintained at this temperature for 4 min. With gaseous samples such as head space volatiles, a splitless injection was used while a 9 : 1 splitting (waste : column) of the injected sample was used with liquid samples. Comparison of the retention time with standards was used to identify the synthesized compounds, while relative peak area was used as a measure of purity.

Heptanal synthesis

The method was adapted from Bradsher and Edgar (1981) with several modifications. The synthesized heptanitrile in CH_2Cl_2 (100 mL) was placed in a round bottom flask, flushed with nitrogen and sealed with a rubber septum. Then it was cooled to −80°C (Bio Freezer, Forma Scientific). The DIBAL-H reagent was cooled to −80°C separately. After three hr, the DIBAL-H reagent was removed from the freezer and cooled in a dry ice (solid CO_2) bath until the reagent was added to the heptanitrile. At the same time, the heptanitrile-containing flask was removed from the freezer and cooled in a liquid nitrogen bath. DIBAL-H (25 mL) was added in 20 min using a 25 mL glass syringe, to the heptanitrile solution while it was being shaken in a liquid nitrogen bath. Using a syringe, nitrogen gas was pumped into the

$$C_6H_{13}CN + H-Al(i-C_4H_9)_2 \longrightarrow \begin{matrix} C_6H_{13} \\ \diagdown \\ \quad C=N-Al(i-C_4H_9)_2 \\ \diagup \\ H \end{matrix} \xrightarrow{H^+/H_2O} C_6H_{13}CHO$$

DIBAL-H reagent bottle, while the reagent was withdrawn from it, to equalize pressure. The reaction mixture was maintained at −80°C for 6 hr. Then, the reaction mixture was removed from the freezer and was placed in a dry ice bath and allowed to

warm gradually while being stirred rapidly for 24 hr. A drying tube was connected to the reaction apparatus through the rubber septum via a syringe. The reaction mixture was extracted with NaF (7g) (Overman and Burke, 1984) in water (50 mL, 4°C), placing the NaF solution in the separatory funnel first. The organic phase was re-extracted with chilled aqueous NaF (3g) solution (4°C, 30 mL), followed by 2% H_2SO_4 (10 mL). For each extraction, adequate time (up to 15 min) was allowed for good phase separation to take place. The organic phase was filtered through anhydrous Na_2SO_4. The organic phase was washed with chilled (4°C) water (5 × 50mL) and each time the organic phase was filtered through anhydrous Na_2SO_4. Anhydrous Na_2SO_4 was used to further dry the combined organic phase. The CH_2Cl_2 (boiling point 39°C) was distilled off very slowly from the organic phase, using a fractionation column. The undistilled fraction was cooled and the purity of the product was analyzed by GC analysis (Varian 3400). Identity of the product was further confirmed by ^{1}H-NMR spectroscopy. The activity of the synthesized ^{14}C-heptanal was analyzed by liquid scintillation counting (Beckman model 8000) using Omni scintillation vials and ScintiVerse liquid scintillation cocktail.

Results and Discussion

A survey of the methods (Larock, 1989) available for the synthesis of heptanal revealed that, due to restrictions imposed by the availability of suitable radiolabeled substrates/reagents, only three synthetic routes were feasible. They were the nucleophilic displacement of alkyl halide by radiolabeled cyanide followed by the conversion of the nitrile to the aldehyde (Winterfeldt, 1975); the conversion of a radiolabeled carboxylic acid to the corresponding alcohol and selectively oxidizing it to the aldehyde

(Collins et al., 1968; Corey and Suggs, 1975); and the reaction of a suitable Grignard reagent with radiolabeled ethyl orthoformate (carboxylic C labeled), followed by hydrolysis to yield the aldehyde (Bachman, 1943). The choice of the first method over the second was based on the ease of separating the radiolabeled reagent ($Na^{14}CN$) from the radiolabeled product ($C_6H_{13}{}^{14}CN$), using the difference in hydrophilicities. In the second method the reagent (RCOOH) and the product (RCH_2OH) have similar solubilities. A further limitation of the second method was that only a few radiolabeled carboxylic acids were commercially available. Due to very low yields, the third method was not pursued any further.

All the glassware was thoroughly cleaned and dried to prevent any interference from foreign matter. Detergents were not used as they were observed to interfere mainly in the conversion of nitrile to aldehyde. The use of 1-iodohexane in place of 1-bromohexane or 1-chlorohexane resulted in higher yield. The temperature was controlled strictly during the synthesis of the nitrile, as higher temperatures turned the reaction mixture dark brown, caused by side reactions with DMSO (Friedman and Shechter, 1960). The heptanitrile distilled at 183°C (2.05g, 85% yield, 98% purity) was pale yellow. Slow distillation favored good separation of components on the fractionation column.

During the preliminary stages of this project several other methods of reducing the nitrile to the aldehyde were attempted. The use of stannous chloride and dry hydrochloric acid (Stephen, 1925) as the reducing agent was abandoned due to the low yields. Reducing agents such as n-tributoxy aluminum hydride and lithium triethoxy aluminum hydride (Brown and Garg, 1964) gave less than adequate yields and isolation of the aldehyde by distillation was difficult. Attempts were made to

isolate the aldehyde from the reaction mixture, by eluting on a silica gel column, by precipitating as the 2,4-dinitrophenyl hydrazone, followed by filtration and hydrolysis to yield the aldehyde. Eluting on the silica gel column was not successful due to the high volatility of the aldehyde. Although the 2,4-dinitrophenyl hydrazone was formed, it was difficult to hydrolyze it and isolate the aldehyde. As such that attempt was not successful.

The reagent DIBAL-H was sensitive to moisture and oxygen, as such the reaction was performed in an apparatus protected from oxygen and moisture. Low boiling CH_2Cl_2 was chosen as the solvent, since it could be distilled off, to separate from the product (heptanal). The other solvents, hexane and benzene, in which the DIBAL-H reagent is available, co-distilled with the heptanal. Freezing the substrate and reagent to $-80°C$ and maintaining them in liquid nitrogen and dry ice, respectively, during the addition of reagent to substrate, enabled the reaction to be started at a very low temperature. The DIBAL-H was not placed in liquid nitrogen as it froze. The syringe used to transfer the reagent was not functional when frozen to $-80°C$, as such it was not frozen. The use of nitrogen gas and a drying tube when needed enabled the removal or addition of liquids from sealed containers and also prevented the reaction apparatus from exploding while warming from $-80°C$ to room temperature. The use of NaF enabled the aluminum salts to be removed as insoluble aluminum fluorides, which rose to the aqueous phase when allowed to stand for 15 min. If the reaction was hydrolyzed without prior removal of the aluminum salts, the whole mixture was entrapped in a gelatinous mass, which hindered further work up. All traces of aluminum had to be washed off to prevent the formation of aldol adducts (as observed during our preliminary studies and confirmed by gas chromatography-mass

spectroscopic analysis). Hydrolysis of the imine complex was completed by H_2SO_4. Filtering through Na_2SO_4, in addition to retaining the moisture, enabled the filtrant that caked on filter paper to be filtered. Slow distillation was the only method of separating the fore runner from the heptanal. Attempts to distill the aldehyde were futile, as it polymerized, turning black. Only a small quantity of heptanal distilled over.

In the method reported here, the light yellow colored heptanal (1.44g, 85% yield) was found to be 90% pure according to GC analysis. The overall conversion of 1-iodohexane to heptanal was 55% and the overall incorporation of the radiolabel was 52%.

During the preliminary stages of this study, attempts were made to synthesize ^{14}C-hexanal, as this synthesized labeled aldehyde was to be used in studying the off-flavor binding to soy protein, and also since hexanal has been identified as a main contributor to the soy off-flavor (Arai et al., 1970a). The method of synthesis used to make hexanal was similar to that reported in this study for the synthesis of ^{14}C-heptanal. The synthesis of hexanal was successful. However, the hexanal could not be separated from the rest of the components in the reaction mixture, because it co-distilled with the fore runner due to its low boiling point of 128°C. Hence, it was decided to attempt the synthesis of another medium chain length aldehyde like heptanal, that had a slightly higher boiling point (152°C) (Weast, 1975).

CHAPTER 3. BINDING OF HEPTANAL TO GLYCININ AND β-CONGLYCININ

The objective of this study was to determine the presence or absence of tightly bound ligands to soy proteins in an aqueous model system. Once the presence of tightly bound ligands was confirmed, attempts were made to determine whether Schiff base formation was the main reason for this tight bonding and/or the other types of bonds responsible for the tight bonding of ligands to glycinin and β-conglycinin.

Materials and Methods

Analytical grade reagents were obtained from commercial sources and used as received. Sodium lauryl sulfate (SDS), N,N,N′,N′-tetramethyl ethylenediamine (TEMED), β-mercaptoethanol (βME), 2[N-morpholino]ethanesulfonic acid (MES), Sepharose 6B-Cl (fractionation range for globular protein 10–4000 kDa), Concanavalin A-Sepharose 4B, Agarose (type 1 : low EEO), methyl α-D-manno-pyranoside (mannoside), N-*tris*[hydroxymethyl]methylglycine (tricine), bovine serum albumin (lyophilized powder), Freund's complete and incomplete adjuvant, urea (electrophoresis reagent), $NaCNBH_3$, iodoacetic acid, N-acetylimidazole, Dalton Mark VI molecular weight standard and Coomassie Brilliant Blue R were purchased from Sigma Chemical Co. (St. Louis, MO). The Dalton Mark VI molecular weight standard contained

a lyophilized mixture of lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), trypsinogen (24 kDa), pepsin (34.7 kDa), egg albumin (45 kDa) and bovine albumin (66 kDa). The chemicals purchased from Fisher Scientific Co. (Fair Lawn, NJ) were *tris*[hydroxymethyl]aminomethane (Tris), sucrose, glycine, acetic acid, sodium azide, calcium lactate, K_2HPO_4, NaCl, sodium potassium tartrate, NaOH, ScintiVerse (liquid scintillation cocktail), Phenol red and ammonium sulfate. Ammonium persulfate (APS), Bromophenol blue, KH_2PO_4, NaH_2PO_4 and $CuSO_4.5H_2O$ were from J. T. Baker Chemical Co. (Phillipsburg, NJ). The MeOH used in the staining and destaining was of practical grade and was purchased from Barton Solvents (Des Moines, IA). The Accugel 40 (40%, acrylamide : *bis* acrylamide = 19 : 1) was purchased from National Diagnostics (Manville, NJ). Blue dextran-2000 and Sephadex G-25-medium were purchased from Pharmacia (Uppsala, Sweden). The *p*-anisaldehyde was from Eastman Kodak Co. (Rochester, NY).

The 0.45 μm filters used for filtration were from Magna (Westborough, MA). The Molecular/Por molecular filtration membranes used for ultrafiltration were from Spectrum Medical Industries Inc. (Los Angeles, CA). Dry micro-Ouchterlony plates were from Sebia (Paris, France). The Biogel Wrap was from BioDesign Inc. (Carmel, NY). The microemulsifying needles were from Thomas Scientific (Swedesboro, NJ). The catheter needles were from Travenol Laboratories Inc. (Deerfield, IL) and the Gel Bond film support medium was from FMC BioProducts (Rockland, ME). The Kimble brand 1 mL borosilicate reaction vials together with the PTFE (polytetrafluoroethylene) lined rubber septa, the aluminum crimp-on seals (11 mm) and the Omni vials (polypropylene scintillation vials) were purchased from Fisher Scientific Co..

Protein purification

Vinton 81 variety soybeans were ground in a coffee and spice mill (Moulinex, France) and were defatted by hexane extraction (soy flour : hexane = 1 : 1.5, v/v) for 2 hr at room temperature. Then the solids were allowed to settle and the solvent was decanted. This was repeated six more times at the end of which the hexane extract was very light yellow. Desolventization of the soy flour was by air drying. The defatted flour was stored at $0 - 5°C$ in plastic bottles until needed.

Isolation of glycinin and β-conglycinin was according to a modified version of the method published by Thanh and Shibasaki (1976). Defatted soy flour (40 g) was stirred for 3 hr in T buffer (0.03 M Tris at pH 8) containing 0.02% NaN_3 and 10 mM βME (800 mL). The solubilized protein was separated from the insoluble matter by filtering through eight layers of cheese cloth, followed by centrifuging. Throughout this procedure a Beckman model J2-21 centrifuge fitted with a JA-17 fixed angle rotor (Beckman Instruments Inc., Palo Alto, CA) was used. The conditions of operation were 10,000 rpm at 20°C for 10 min. The pH of the supernatant was adjusted to pH 6.1–6.4, stirred for 1 hr, and centrifuged to separate out the precipitated crude glycinin. The precipitated crude glycinin was dissolved in the minimum amount of Wolf's buffer (30 mL) (0.0026 M KH_2PO_4, 0.0325 M K_2HPO_4, 0.4 M NaCl, 0.01 M βME and 0.02% NaN_3 at pH 7.4) and was used for further purification. The pH of the supernatant was adjusted to 5.5, stirred for 1 hr and centrifuged. The supernatant was adjusted to pH 4.8, stirred for 30 min and centrifuged to separate the precipitated crude β-conglycinin. The precipitated crude β-conglycinin was redissolved in the minimum amount of Wolf's buffer (40 mL), adjusted to pH 7.0, stirred for 30 min and centrifuged. The supernatant was used as the crude β-conglycinin sample.

The crude glycinin was purified on a Sepharose 6B-Cl column (3.2×85 cm). The eluent was Wolf's buffer and 20 mL of crude glycinin was loaded onto the column at a time. The glycinin containing peak was concentrated by ultrafiltration. The retentate was eluted on a Concanavalin A-Sepharose 4B (3.2×22 cm) column (Kitamura et al., 1974) to remove any β-conglycinin contaminants present. The glycinin did not bind to the Concanavalin A and was eluted close to the void peak. The β-conglycinin bound to the Concanavalin A column was eluted out by washing with 0.01 M mannoside in Wolf's buffer. The column was equilibriated in Wolf's buffer before reuse. The purified glycinin was concentrated by ultrafiltration and stored at 0–5°C until needed.

The crude β-conglycinin was purified by elution on a Concanavalin A-Sepharose 4B column (3.2×22 cm). A 30 mL sample was loaded at a time and the eluent was Wolf's buffer. After the contaminants were washed off, the eluent was changed to 0.01 M mannoside in Wolf's buffer to elute the bound β-conglycinin. The eluted β-conglycinin was concentrated by ultrafiltration and stored at 0–5°C until needed. The column was equilibriated in Wolf's buffer before reuse. After the elution of four samples of β-conglycinin, the column was washed in 0.05 M mannoside to regenerate the capacity of the column.

Before being used in the binding studies, the proteins (protein : buffer = 1 : 10) were ultrafiltered with the T buffer twice, stored at 4–5°C and their concentration, purity and immunoreactivity were analyzed. All the buffers were filtered through a 0.45μm filter before use. The elution pattern of each column purification was monitored by absorbance measurements at 280 nm. The elution rate was 10 drops/min and 10 mL fractions were collected. Fractions corresponding to the same peak were combined and the presence of the protein of interest was determined by micro-

Ouchterlony analysis. The dry Ouchterlony plates were hydrated in Wolf's buffer and the antigen was placed in the center well while the antibody was placed in the peripheral wells. Immunodiffusion was carried out for 16 hr at room temperature in a moist atmosphere. Unreacted material was washed out by shaking in 0.15 M NaCl for 30 min. It was then stained in Coomassie blue stain for 2 hr and then destained in a solution consisting of acetic acid : methanol : water = 1 : 5 : 4. When needed, samples were ultrafiltered at 20 psi pressure of nitrogen in a ultrafiltration apparatus (Nuclepore, Pleasanton, CA) fitted with a magnetic stirrer. With glycinin and β-conglycinin samples, the ultrafiltration membranes used had molecular weight cut-off at 50 kDa and 10 kDa, respectively. Effectiveness of purification of the glycinin and β-conglycinin samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Determination of protein concentration

The protein concentration was determined by the Biuret method where a Cu^{2+} ion in alkaline solution, complexes with peptide linkages to give a red color that has an absorbance maximum at 540 nm. The Biuret reagent consisted of 1.5 g/L $CuSO_4.5H_2O$, 6 g/L sodium potassium tartrate and 30 g/L NaOH. Several dilutions of a standard protein solution (10 mg/mL bovine serum albumin) were made in water to a final volume of 1 mL. The protein solutions being analyzed were also diluted accordingly to a final volume of 1 mL. The Biuret reagent (4 mL) was added to the protein, mixed well and allowed to stand for 30 min. The absorbance was measured at 540 nm in a Gilford spectrophotometer 250 (Gilford Instrument Laboratories Inc., Elgin, IL). Using a Beer's law plot, the concentration of the proteins was determined.

Determination of protein purity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the purity of the glycinin and β-conglycinin samples. The method was adapted from Laemmli (1970) with a few modifications. A discontinuous gradient gel was used. The stacking gel was a non–restrictive gel with 4% acryl-*bis*, 0.1% SDS, 0.065% TEMED, 0.1% APS and 0.125 M Tris at pH 6.8. The resolving gel was a linear gradient with acryl-*bis* concentration varying from 10–15% with 0.1% SDS, 0.065% TEMED, 0.1% APS and 0.375 M Tris at pH 8.8. A $16 \times 14.2 \times 0.15$ cm slab gel was prepared with a 12 cm resolving gel and a 4 cm stacking gel in which 15 wells were made by the use of a comb. The sample was prepared for loading onto the gel by boiling in a water-bath for 10 min together with tracking dye and βME (protein solution : tracking dye : βME = 10 : 2 : 1 by volume). The tracking dye consisted of 5% SDS, 0.5 g/mL sucrose, 0.05 M MES and adequate Bromophenol blue to give a dark blue coloration. The slab gel was assembled on a Sturdier slab gel electrophoresis unit model 400 (Hoefer Scientific Instruments, San Francisco). The samples were loaded into the wells with only tracking dye in the end wells, and the apparatus was assembled for electrophoresis with tank buffer in both upper (300 mL) and lower (200 mL) buffer chambers. The tank buffer consisted of (25 mM) Tris, (192 mM) glycine, and (0.1%) SDS. The pH of the tank buffer was 7.9. A sample of whole soy protein extract and a sample of Dalton Mark VI molecular weight standard were run in two lanes so that these could be used as references. The samples were electrophoresed at constant current (38 mA) for 4.5 hr or till the dye was 0.5 cm from the bottom of the gel. Upon completion of electrophoresis, the gel was removed from the slab and stained in staining solution (0.1% Coomassie blue in strong destain

solution). The strong destain solution consisted of MeOH:acetic acid:water = 5:1:4 volume basis. The staining was performed while shaking gently for 16 hr. Then the stained gel was destained for 24–36 hr in 2–3 changes of strong destain until the background was very light. The gel was gently shaken in the destaining solution to enhance the destaining process. The gels were visually examined with reference to the positioning of the protein bands in the whole soy protein extract. When it was necessary to store the gel for a shorter period of time, it was kept in strong destain solution closed in a plastic box. For long term storage, the gel was dried between two layers of Biogel Wrap.

Antibody production

The method of antibody production was adapted from Mayer and Walker (1980). Purified glycinin and β-conglycinin were used for this purpose and three month old male goats were used, one for each protein. Prior to the first inoculation of the antigen, a sample of blood (5 mL) was removed from the jugular vein of the goats and checked for the presence of any antibody to either of these proteins. The antigen (0.25 mg) together with 0.5 mL Freund's complete adjuvant was made up to 1 mL in 0.7% NaCl solution to be injected into the goat each time. Prior to injection, the innoculum was emulsified between two syringes connected by a microemulsifying needle. This protein emulsion was inoculated subcutaneously once every two weeks for four times. After that Freund's incomplete adjuvant was used in place of the complete adjuvant. Boostings were done on a monthly basis and the amount of protein injected was reduced to 0.1 mg/injection per goat. Starting at the age of 5 months, blood samples were tested by Ouchterlony for the presence of a good titer.

Once the titer was high, blood samples were withdrawn from the jugular veins of the goats, starting at 150 mL and gradually increasing to 250 mL volume of blood per goat, every two weeks. When over 50 mL of blood was withdrawn per goat per time, a catheter needle was used to bleed the goat.

The serum was obtained from the blood by ringing the blood and allowing it to clot at room temperature, overnight. The serum was separated by centrifuging at 10,000 rpm at 15°C for 15 min. The serum was decanted off and stored at 0–5°C until about 1 L of serum was collected. The serum (1 L) was thawed and during the process of isolating the antibody it was maintained in an ice bath. The antibody was precipitated by a 50% $(NH_4)_2SO_4$ cut (291 g $(NH_4)_2SO_4$ per L serum). The precipitate was separated by centrifuging at 10,000 rpm at 2°C for 10 min. The precipitate was washed about seven times (until the washings were clear) with 1.75 M $(NH_4)_2SO_4$, centrifuging between washings to separate the precipitate from the supernatant. At the end of the washing, the precipitate was dissolved in minimum quantity of 10 mM NaH_2PO_4 at pH 7 (30 mL) and dialyzed against water overnight at 4–5°C. It was centrifuged at 10,000 rpm at 2°C for 10 min to remove the lipoproteins that had precipitated. The supernatant was dialyzed against 10 mM NaH_2PO_4 at pH 8 to get the antigen. This purified antibody was stored at 0–5°C until needed.

Rocket immunoelectrophoresis

This method of electrophoresis could be used to quantitate the amount of glycinin and β-conglycinin in a whole soy protein extract. In this study it was also used to check for the presence of native structure of the protein being tested, in the same amount determined by the Biuret method. It was also used to check for the absence

of β-conglycinin in the glycinin sample and vice versa.

A gel cast was assembled to make a 18 × 9 × 0.15 cm gel with two glass plates, 3 acrylic strips and a Gel Bond film which was aligned so that the hydrophilic side faced the gel. Agarose (300 mg) was heated while stirring on a water bath, in rocket electrophoresis buffer (0.025 M tricine, 0.08 M Tris, 0.34 mM calcium lactate and 0.02% NaN_3 at pH 8.6) (29 mL) to 90–95°C, to solubilize the agarose. The mixture was cooled to 55°C and either glycinin or β-conglycinin antibody (1 mL) was added. This mixture was poured into the gel cast using a syringe, while at a temperature between 50–55°C. Both the gel cast and the syringe were warmed to about 50°C under a heat lamp to prevent the gel from sudden cooling upon contact with it. The gel was allowed to set at room temperature for 30 min. The gel was removed from its cast, and 35 wells were made (1 cm from the bottom edge) using a steel tube of diameter 3 mm (Pratt and Witney cutting tools and gages) and the cut out gel was sucked out of the wells. The gel was adhered to the stage of the electrophoresis unit (Model 1405 electrophoresis cell, Biorad Laboratories, Richmond, CA) using a few drops of water. The wells were loaded with standard and unknown protein samples (4–5 μL) in a random order. The protein being tested and the standards used were determined by the antibody incorporated into the gel. Rocket electrophoresis buffer (total 500 mL) was placed in both the buffer chambers, contacts were established between the buffer and the gel using filter paper and the electrophoresis unit was set up for electrophoresis. The electrophoresis was at 180 mV for 16 hr. At the end of the electrophoresis run, the gel was removed from the unit, dried between filter papers and absorbent paper (10 min), pressed under a weight of 1000 g and rinsed in 0.1 M NaCl for 10 min. The gel was rinsed in water (2×) for 5 min each, pressed again and

dried under a heat lamp for 15 min. It was stained for 15–20 min in 0.5% Coomassie blue in strong destain and then destained in strong destain until the background was clear. A calibration plot of height of rocket vs μg standard protein was used to determine the concentration of the protein being tested, in the samples analyzed.

General procedures used in the binding experiments

A gel filtration technique, which was a modification of the Hummel and Dreyer (1962) method, was used to determine the ^{14}C-heptanal bound to glycinin and β-conglycinin. The ligand was not incorporated in the mobile phase, as the ^{14}C-heptanal ligand used was available only in small quantities. The concentration of the components in the binding assay mixture were similar to those used by O'Keefe et al. (1991ab), as the present study was a continuation of the investigations performed by O'Keefe et al. (1991ab).

The gel filtration medium of choice was Sephadex G-25-medium, as it had a molecular weight cut–off at 5 kDa, and was a gel that behaved as rigid spheres in gel filtration. Three glass columns (diameter 12 mm, lengths 8, 50 and 100 cm) were made in the Glass Shop at Iowa State University. These columns had coarse glass fritt bed supports, pipette tip outlets and were fitted with teflon stop–cocks to control the flow rate. Unless otherwise mentioned, the buffer used in the binding assay solutions and that used as the eluent was the T buffer (0.03 M Tris at pH 8). This buffer did not contain any reducing agent or preservative. Two glass syringes with fixed steel needles (Hamilton Co., Reno, NV) of graduated volumes 10 μL and 1 mL, were used to transfer ^{14}C-heptanal solution and the binding assay solution, respectively. Upon elution, the eluate was collected in 0.5 mL fractions into 4 mL

scintillation vials containing cocktail (1.5 mL), then the vials were filled with cocktail (1.5 mL) stoppered and mixed thoroughly. The radioactivity was analyzed in a liquid scintillation counter (Beckman model 8000). A completely randomized experimental design was used and all the analyses were performed in triplicate. A Scheffe s test was performed on the data to test for significant differences between column lengths and modifications of the protein surface.

Prior to eluting the binding assay mixture on the column, an aqueous mixture of Blue dextran-2000 and Phenol red was eluted on this column with T buffer eluent. Fraction numbers in which the Blue dextran and the Phenol red eluted were noted as the approximate fractions in which the protein and the aldehyde were expected to elute, respectively. During the preliminary studies, the absorbance of the column elution was monitored at 280 nm to determine the position of elution of the protein. The radioactivity counting was used to enumerate the protein–bound aldehyde that co–eluted with the protein peak and the free aldehyde that eluted later. For the purpose of this study, the molarity of the protein solutions were calculated using molecular weights of 320 and 160 kDa for glycinin and β-conglycinin, respectively. Since there is some discrepancy in the literature, regarding the value of the molecular weight of these proteins, these values were chosen in keeping with the values used by O'Keefe et al. (1991ab).

Determination of binding

The purified protein solution (glycinin or β-conglycinin) and the ^{14}C-heptanal solution were equilibriated to room temperature and 10 mg of the protein was placed in each 1 mL reaction vial. A 1000 ppm solution of ^{14}C-heptanal in T buffer was

prepared by diluting 1 μL ligand to 1 mL. This solution was used as the stock solution to add the ligand for the binding assay concentrations ranging from 100 ppm to 700 ppm. A 2000 ppm ^{14}C-heptanal stock solution was made to use for making the binding assay concentrations 750 ppm to 1000 ppm. At times 1 μL of ^{14}C-heptanal was added directly to make the 1000 ppm binding assay concentration. Suitable volumes of ^{14}C-heptanal or ^{14}C-heptanal stock solution were added to the protein solution, to make a series of binding assays consisting of ^{14}C-heptanal concentrations ranging from 100 ppm to 1000 ppm. The assay mixture containing protein and ligand was brought up to a volume of 1 mL by adding T buffer. The seals with the septa were crimped-on (Supelco Inc., Bellefonte, PA) tightly and the reaction vials were shaken on a wrist action shaker (Burrell Corporation, Pittsburgh, PA) for two hr.

A size exclusion column of Sephadex G-25-medium of bed length 6 cm (in the 8 cm column) was equilibriated in T buffer. A 100 μL sample of the binding assay mixture was loaded onto this column and eluted with T buffer at a rate of 10 drops/min. The eluate was collected in 0.5 mL fractions and the radioactivity was measured. The radioactivity in the protein-bound aldehyde peak and in the free aldehyde peak was used to determine the amount of aldehyde bound to the protein, and the amount of free aldehyde. These data were used to construct a saturation curve. The experiments were repeated using a 4 cm Sephadex column.

Exhaustive elution of the protein–ligand complex

A binding assay solution (1 mL) consisting of 1% protein (glycinin or β-conglycinin) and 1000 ppm ^{14}C-heptanal in T buffer was equilibriated for two hr. A column was packed to a bed length of 45 cm with Sephadex G-25-medium gel filtration

medium and was equilibriated in T buffer. Of the binding assay solution, a sample (50 μL) was analyzed separately for radioactivity and another sample (900 μL) was eluted on this Sephadex column with T buffer. Eluted fractions (0.5 mL each) were collected and the radioactivity was analyzed to determine the amount of ^{14}C-heptanal still bound to the protein. The experiment was repeated with a Sephadex G-25-medium column of 90 cm bed length.

Effect of reducing agent on ligand binding to protein

The binding assay (1 mL) consisted of 1% protein (glycinin or β-conglycinin), 1000 ppm ^{14}C-heptanal, 5 M urea and 20 mM $NaCNBH_3$ in T buffer. The reaction mixture was equilibriated at 4–5°C for 16 hr before elution. Of the binding assay solution a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements.

Effect of protein unfolding on ligand binding to protein

The binding assay solution (1 mL) consisting of 1% protein (glycinin or β-conglycinin), 1000 ppm ^{14}C-heptanal and 5 M urea in T buffer was equilibriated for 2 hr. Of this solution a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The eluent used was 2.5 M urea in T buffer. The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements. A similar experiment was performed to check for the effect of omission of urea from the eluent. The experimental set up was the same as above other than the fact that the eluent did not have any urea

incorporated in it.

Effect of SDS and protein unfolding on ligand binding to protein

The binding assay solution (1 mL) consisted of 1% protein (glycinin or β-conglycinin), 1000 ppm ^{14}C-heptanal, 5 M urea and 1% SDS in T buffer. Of the binding assay solution a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The eluent used was 2.5 M urea and 1% SDS in T buffer. The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements.

Effect of protein modification on the binding of ^{14}C-heptanal to protein

Effect of blocking the amino groups of the protein The method of reductive methylation of the ϵ-amino group of lysine residues was adapted from the method published by Jentoft and Dearborn (1979). Anisaldehyde was chosen as the aldehyde and $NaCNBH_3$ was the reducing agent. The choice of anisaldehyde as the aldehyde was based on the fact that it was deemed GRAS (generally recognized as safe) by the FEMA (Flavor Extract Manufacturers Association) (Furia, 1968) as a food additive. $NaCNBH_3$ was a mild reducing agent that was capable of reducing Schiff bases.

$$\text{(P)} - NH_2 + R - CHO \longrightarrow \underset{\text{Schiff base}}{\text{(P)} - N = CH - R} \xrightarrow{NaCNBH_3} \text{(P)} - NH - CH_2R$$

The binding assay solution (1 mL) contained 1% protein (glycinin or β-conglycinin), 2000 ppm anisaldehyde, 5 M urea and 20 mM $NaCNBH_3$ in T buffer. This mixture was equilibriated at 4–5°C for 16 hr, then ^{14}C-heptanal (1 μL) was added

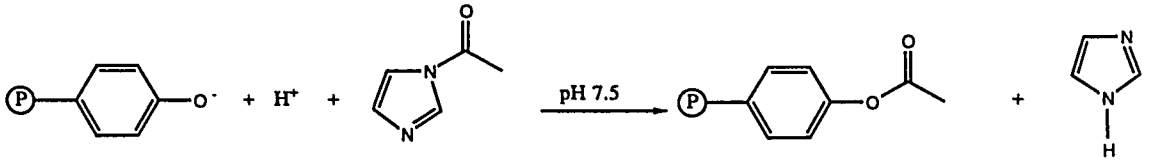
to give a concentration of 1000 ppm and it was shaken at room temperature for 2 hr. Then a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements.

Effect of blocking the sulfhydryl groups The method of S-carboxymethylating cysteine was similar to the method published by Cole et al. (1958). A binding

$$\textcircled{P} - S^- + ICH_2COO^- \xrightarrow{pH \geq 7} \textcircled{P} - S - CH_2COO^- + I^-$$

assay solution (1 mL) containing 1% protein (glycinin or β-conglycinin), 0.16% iodoacetic acid and 5 M urea in T buffer was equilibriated at room temperature for half an hr, then ^{14}C-heptanal was added to give a concentration of 1000 ppm and it was shaken for 2 hr. After that a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements.

Effect of blocking the tyrosyl group The method of O-acetylation of the tyrosyl residues was the method used by Simpson et al. (1963) and Riordan et al. (1965). In the method reported by these researchers, a 60–fold molar excess of N-acetylimidazole was used at pH 7.5 and room temperature. After one hr the reaction was stopped by eluting on a Sephadex G-25 column. In the present study,



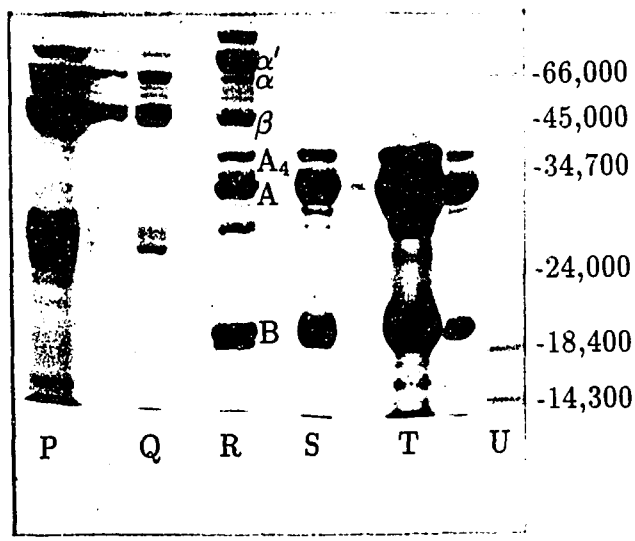
the reaction was performed at pH 8 (as the rest of the experiments were done at this pH). Approximately 0.5 g of protein (glycinin or β-conglycinin) in T buffer with 5 M urea was reacted with a 60–fold molar excess of N-acetylimidazole for one hr at room temperature. The reaction was stopped by separating the reagents from the protein by ultrafiltration through a 10 kDa membrane. This O-acetylated protein was used to determine the effect of blocking the tyrosyl group, on ligand binding to soy protein.

A binding assay solution (1 mL) was set up with 1% O-acetylated protein (glycinin or β-conglycinin), 5 M urea and 1000 ppm ^{14}C-heptanal in T buffer. The assay was equilibriated at room temperature for 2 hr. After that a sample (50 μL) was analyzed for radioactivity and a sample (900 μL) was eluted on a Sephadex G-25-medium column (length 90 cm). The ^{14}C-heptanal bound to the eluted protein was determined by radioactivity measurements.

Results and Discussion

Preparation of protein for the binding study

The method of isolating glycinin and β-conglycinin was very effective. As illustrated in Figure 3.1, the SDS-PAGE indicated the level of purity of the two isolated protein samples. The purpose of electrophoresing an overloaded sample of glycinin and β-conglycinin was to illustrate its purity and the absence of contaminants. Immunoelectrophoretic analysis of a glycinin sample on a β-conglycinin rocket gel and vice versa, illustrated the absence of cross contamination of the two protein samples. Rocket electrophoretic analysis showed that the isolated glycinin and β-conglycinin samples were not denatured and that the protein concentrations were



α', α, β	-	subunits of β−conglycinin
A_4, A	-	acidic chains of glycinin
B	-	basic chain of glycinin
P,Q	-	β−conglycinin
R	-	whole soy protein
S,T	-	glycinin
U	-	Dalton Mark VI molecular weight standard

Figure 3.1: Purity of the isolated glycinin and β-conglycinin as illustrated by SDS-PAGE

similar to that determined by Biuret test.

Determination of binding

The protein–bound aldehyde peak and the free aldehyde peak were not adequately separated in the data obtained on the 4 cm column (data not shown), as such these data were discarded. Attempts were made to construct saturation curves from the data obtained for the amount of protein–bound aldehyde and free aldehyde, for the series of concentrations of the binding assay, eluted on the 6 cm column (Figure 3.2a). According to these plots, saturation was not reached with ^{14}C-heptanal binding to glycinin. However, with β-conglycinin it seems that saturation may have been reached at about 25 mol bound heptanal/mol protein. At this stage, attempts were made to increase the range of ^{14}C-heptanal used in the binding study, hoping to study the binding of ^{14}C-heptanal with glycinin until saturation was reached. Investigating the binding of ^{14}C-heptanal to β-conglycinin would have served to confirm whether saturation was truely reached. However, at a concentration of 1100 ppm ^{14}C-heptanal, phase separation was observable, indicating that ^{14}C-heptanal could not be maintained in solution at this concentration in the binding assay solution.

According to Klötz (1982), if saturation was truely reached, then, when these same data are plotted on a semilogarithmic scale (bound ligand vs log free ligand), it should result in an S-shaped curve. The semilogarithmic curves are shown in Figure 3.2b. These plots clearly illustrate that saturation was not reached with glycinin. Inaddition, the β-conglycinin does not unequivocally demonstrate the S-shaped curve expected if saturation was reached. Thus, it was concluded that it was unreliable to reach a conclusion on the total number of binding sites for ^{14}C-heptanal

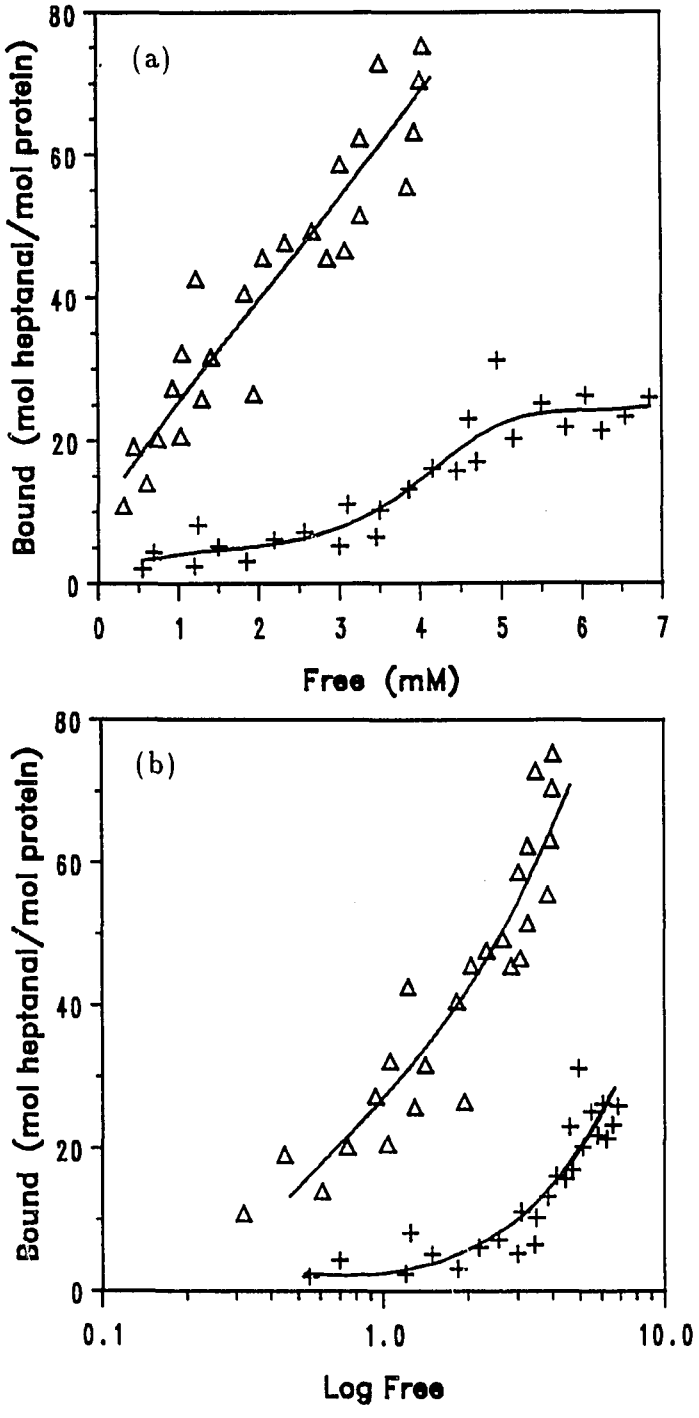


Figure 3.2: Binding of ^{14}C-heptanal to glycinin ($\Delta\Delta$) and *3*-conglycinin ($+-+$) (a) saturation curve; (b) semilogarithmic curve

binding to either glycinin or β-conglycinin, from these data.

Although it was not possible to conclude on the total number of binding sites for ^{14}C-heptanal binding to glycinin and β-conglycinin, these data clearly illustrate that at the ligand concentration range investigated, glycinin bound more heptanal per mol protein than β-conglycinin, and that both these proteins had high binding capacities for heptanal. Next, attempts were made to verify whether aldehyde binding to soy proteins was completely reversible.

Reversibility of ^{14}C-heptanal binding to soy proteins

A method of exhaustive elution of the protein–ligand complex on a size exclusion column (45 cm or 90 cm) with an eluent that did not contain the ligand ^{14}C-heptanal in it was used for this purpose. This Sephadex G-25-medium size exclusion column used, had the ability of separating small molecules (^{14}C-heptanal) from large molecules, by excluding the large molecules from the gel and allowing the smaller molecules to traverse through the gel (the molecular weight cut-off of the Sephadex G-25-medium gel was 5 kDa). Hence, during the process of elution, the column constantly separated the free ligand from the protein and the protein–ligand complex. As a result, the equilibrium between the protein and the ligand, as illustrated in the following equation, should have shifted to the left.

$$\text{protein} + \text{ligand} \rightleftharpoons \text{protein}-\text{ligand complex}$$

If the binding was completely reversible, it should be possible to drive the above equilibrium to the left by exhaustive elution, until all the ligand was free (no more ligand existed in protein–bound form).

A 1000 ppm ligand concentration was chosen for the binding assay, so that the

radioactivity of the fractions eluted from the column (45 cm or 90 cm) would be high enough to be analyzed with reasonable accuracy. A binding assay containing 1000 ppm ^{14}C-heptanal was eluted on the 45 cm column and then a similar sample was eluted on a 90 cm column. As illustrated in Table 3.1, there was a reduction in the amount of protein–bound ligand when going from a 6 cm column elution to a 45 cm column elution, but when changing from a 45 cm column to a 90 cm column, there was only an insignificant change. Thus, it seems that the amount of ligand bound to the protein, after eluting on a 45 cm column, was not very easy to separate from the protein. This fraction of ^{14}C-heptanal that could not be dissociated from the protein upon eluting on a 90 cm Sephadex column was categorized as "tightly bound ligand". Cantley and Hammes (1975) defined "tightly bound ligand" as the ligand that remained bound to the protein upon elution on two Sephadex columns, each 50 cm long. These data also indicate that some of the ^{14}C-heptanal bound to soy proteins were probably very weakly bound and thus were easily dissociated from the protein upon eluting on a 45 cm column.

Table 3.1: Effect of Sephadex G-25-medium column length on the dissociation of the soy protein-^{14}C-heptanal complex

Protein	^{14}C-heptanal bound to protein upon elution on different length columns (mol heptanal bound/mol protein)		
	6 cm	45 cm	90 cm
glycinin	68.3 ± 7.6^a	7.6 ± 1.3^b	7.5 ± 1.3^b
β-conglycinin	23.1 ± 4.3^c	9.1 ± 2.4^d	7.0 ± 1.0^d

^{a,b,c,d}values with same letter are not significantly different according to Scheffe s test ($\alpha = 0.05$).

In order to test for the possibility of some ligand being physically entrapped within the protein, a binding assay was set up with 5 M urea in the assay mixture. A 5 M concentration of urea was reported to be able to denature protein by disrupting hydrogen bonding (Hames and Rickwood, 1990). In order to maintain the protein in denatured state throughout the elution on the 90 cm Sephadex column, 2.5 M urea was incorporated into the eluent. As shown in Table 3.2, unfolding the protein did not reduce the amount of ligand bound to the protein. This indicated that ligand entrapment within the protein was not a major contributor to the tight binding of ^{14}C-heptanal to soy proteins. However, these data were not adequate to exclude the existence of any entrapment of ligand in the binding of ^{14}C-heptanal to soy proteins. The similarity of the results obtained when the binding assay was treated with urea, either with or without incorporating urea into the eluent, indicated that the effect of incorporating urea into the eluent was not significant. As such urea was not incorporated into the eluent when the amino acid residues were blocked, as urea tended to block the column.

Having established that ^{14}C-heptanal binding to soy proteins was not completely reversible, attempts were made to identify type/types of bonds involved in the tight interaction between ^{14}C-heptanal and soy proteins.

Determination of the types of bonds involved in the tight interaction of ^{14}C-heptanal with soy proteins

The amount of ^{14}C-heptanal still bound to the protein upon eluting the modified protein on a 90 cm Sephadex column are given in Table 3.2. Urea unfolds the protein, exposing the parts of the polypeptide chain that were buried within the interior of the

Table 3.2: Effect of modification of the protein on the amount of ^{14}C-heptanal tightly bound to glycinin and β-conglycinin upon elution on a 90 cm Sephadex G-25-medium column

Modification	^{14}C-heptanal bound per mol protein	
	glycinin	β-conglycinin
control[a]	7.5 ± 1.3^{k}	7.0 ± 1.0^{o}
urea[b]	30.5 ± 0.9^{g}	10.0 ± 0.6^{m}
urea[c]	31.2 ± 0.5^{g}	9.0 ± 0.5^{m}
SDS[d]	3.0 ± 0.8^{h}	1.0 ± 0.4^{n}
$NaCNBH_3$[e]	47.9 ± 1.0^{i}	13.2 ± 1.2^{m}
$NaCNBH_3$ and anisaldehyde[f]	14.3 ± 1.8^{j}	9.0 ± 1.8^{m}
iodoacetic acid	10.0 ± 1.0^{jk}	3.1 ± 1.1^{no}
N-acetylimidazole	41.5 ± 1.4^{l}	12.7 ± 2.0^{m}

[a]Untreated binding assay.

[b]5 M urea was incorported into the binding assay only.

[c]5 M urea was incorporated into the binding assay and the eluent contained 2.5 M urea.

[d]The binding assay contained 5 M urea and 1% SDS while the eluent had 2.5 M urea and 1% SDS.

[e]The binding assay contained 20 mM $NaCNBH_3$ in addition to 1000 ppm ^{14}C-heptanal, 1% protein and 0.03 M T buffer.

[f]The binding assay contained 20 mM $NaCNBH_3$ as well as 2000 ppm anisaldehyde.

[g,h,i,j,k,l,m,n,o]values with the same letter are not significantly different according to Scheffe s test ($\alpha = 0.05$).

native protein. Both glycinin and β-conglycinin have been identified as proteins with high levels of β-sheet structure and low levels of α-helix structure (Plietz et al., 1987; Dev et al., 1988; Deshpande and Damodaran, 1990). It is difficult to predict whether an amino acid will favor an α-helix, β-sheet or another secondary structure. Further, an amino acid that does not favor a certain type of secondary structure, may occur in that secondary structure due to its positioning in the amino acid sequence of the polypeptide. However, by studying the relative frequencies of occurrence of amino acid residues in the secondary structure of proteins (Creighton, 1983), it has been shown that the amino acid residues that occur most frequently in β-sheet structure are the hydrophobic amino acids valine, isoleucine, phenylalanine, tryptophan and tyrosine. Hence, it seems that both these proteins have a high content of hydrophobic amino acids.

According to the data presented in Table 3.2, the amount of ^{14}C-heptanal bound to the soy proteins increased upon unfolding of the protein by urea. In proteins, most of the hydrophobic amino acid residues are buried inside the native structure (Tanford, 1973). Upon unfolding of the protein by urea, these hydrophobic amino acids become exposed. Since these hydrophobic groups are not soluble in the aqueous medium, the unstability of these hydrophobic groups causes a hydrophobic effect. In the presence of a hydrophobic ligand, this hydrophobic effect would favor the binding of the hydrophobic ligand to the exposed hydrophobic amino acid residues. Thus, unfolding of the protein polypeptide chain by urea, exposes more hydrophobic groups and thereby increases the hydrophobic surface available for interacting with hydrophobic ligands. This indicates that hydrophobic interactions may be involved in the tight bonding of ^{14}C-heptanal to soy proteins.

SDS caused a drastic decrease in the amount of ^{14}C-heptanal bound to soy proteins down to almost none. SDS is an anionic detergent, which has a long hydrophobic group with an anionic group at one end. SDS binds to proteins through its hydrophobic group and the sulfate group remains exposed to the aqueous medium. In fact, by investigating the binding of SDS, sodium decyl sulfate, tetradecyl sulfate and alkanes, to β-lactoglobulin A, Robillard and Wishnia (1972) have shown that SDS binds to the same binding site as alkanes bind and that the binding is hydrophobic in nature. This postulation of the involvement of hydrophobic interaction in the binding of SDS to proteins was supported by Kato et al. (1984), who studied the binding of SDS to several proteins including soy glycinin and β-conglycinin. Interestingly, the SDS binding capacity changed corresponding to the surface hydrophobicity of the protein and was not influenced by a change in ionic strength of the medium. If electrostatic bonding was involved in the SDS binding to proteins, then the SDS binding capacity should have decreased with an increase in ionic strength. As such, these data accentuated the importance of hydrophobic interactions in the binding of SDS to protein. In the experiment designed to study the effect of SDS on the binding of ^{14}C-heptanal to soy protein, the protein was equilibriated with 1000 ppm aldehyde, 5 M urea and 1% SDS and then eluted on the column using T buffer that contained 2.5 M urea and 1% SDS. As explained earlier, urea unfolds the proteins. The 1% SDS, in addition to helping to maintain the protein in the unfolded state, binds to the unfolded polypeptide chain, and makes the surface negatively charged. Since heptanal is an electron deficient molecule, if the interaction between the heptanal and the soy proteins were electrostatic in nature, then the heptanal should have been drawn to the protein–SDS surface, due to the negative charge resulting on the protein surface, as a result of SDS

binding. Upon eluting the protein ligand complex on a 90 cm Sephadex column, the amount of ligand still bound to the protein was negligible. Even if there were any ^{14}C-heptanal bound to the protein–SDS surface, it seemed to have been very loosely bound, and would have been reversibly dissociated from the protein–SDS surface, upon exhaustive elution on a 90 cm Sephadex column. Hence, this experiment also supports the postulation that the interaction causing tight bonding of heptanal to soy proteins is in fact hydrophobic in nature.

SDS has a longer hydrophobic chain than heptanal. Thus, the hydrophobic chain of SDS would be destabilized to a greater extent in an aqueous medium than the hydrophobic chain of heptanal, due to the hydrophobic effect. Since the SDS was used in a higher concentration than heptanal in the binding assay solution, and SDS was also used in the eluent, the SDS was capable of out-competing the heptanal for binding to the hydrophobic surface of the unfolded protein, on concentration basis. Further, the hydrophobic effect would force the SDS, to a greater extent than the heptanal, to aggregate with the hydrophobic surface of the unfolded protein. As a result, the amount of aldehyde still bound to the protein upon exhaustive elution, was almost negligible.

In order to investigate the involvement of Schiff bases in the tight bonding of ^{14}C-heptanal to soy proteins, a method of fixing the Schiff bases via reduction was used. Once the Schiff base is reduced, the aldehyde would be tightly (covalently) bound to the protein. This would also be a form of tight bonding that would not be dissociated by exhaustive elution. As seen in Table 3.2, the amount of aldehyde still bound to the soy proteins was greater than that bound to the unfolded protein upon elution under conditions that favor unfolding. This means that reducing the Schiff

bases did make some aldehyde molecules that were not tightly bonded in the absence of $NaCNBH_3$, to be tightly bonded to the protein in the presence of $NaCNBH_3$. These data suggest that Schiff base formation was not involved in tight bonding of the aldehyde to the protein. However, these data do not suggest that Schiff base formation is not involved in the interaction between aldehyde and soy protein. During the process of exhaustive elution, any aldehydes bonded by Schiff base formation to soy protein would have been reversibly dissociated from the protein and thus did not contribute to the fraction of aldehyde that was considered tightly bound.

Next, attempts were made to block the exposed amino groups by first reacting the protein with another aldehyde in the presence of a reducing agent, before using the proteins in the binding assay. Anisaldehyde was used as the other aldehyde. This resulted in a great reduction in the amount of ^{14}C-heptanal bound tightly to the soy proteins. The amount tightly bound was less than that observed in the experiment used to study the effect of urea. Hence, anisaldehyde seemed to have not only blocked the amino groups (by bonding to them), but also may have bonded to some hydrophobic sites of the polypeptide chain. However, anisaldehyde being a larger aldehyde than heptanal, would have permitted heptanal to bind to some hydrophobic binding sites that were too small for anisaldehyde to interact with.

Reaction with iodoacetic acid reduced the amount of ^{14}C-heptanal tightly bound to a level well below the amount bound to the protein upon unfolding the protein with urea. This means that reaction with iodoacetic acid has decreased the amount of binding sites that were available on the unfolded polypeptide chain. Iodoacetic acid is known not only to react with the sulfhydryl groups, but also to react with the ϵ-amino groups of lysine to a lesser extent. Reaction with iodoacetic acid leaves the

protein surface negatively charged. Thus, a situation similar to that occurring in the presence of SDS is occurring here too. The carboxylic group of iodoacetic acid should be ionized at pH 8 (pK_a = 3.0–3.2, Fromm, 1975). Although heptanal is an electron deficient molecule, it does not seem to bond to the negatively charged carboxylic group (in the acetate group) introduced to the protein by reacting with iodoacetic acid. However, reaction with iodoacetic acid does not make the whole surface negatively charged, and there would be some hydrophobic sites to which the ^{14}C-heptanal was able to bind tightly. Since making the protein surface more charged reduced the amount of aldehyde tightly bound, these data also support the postulation that tight binding of aldehydes to soy proteins were caused by hydrophobic bonding. Blocking the sulfhydro groups did not eliminate the tight interaction between ^{14}C-heptanal and soy proteins. As such chemical bonding with the sulfhydro groups does not seem to be a major contributor to the tight bonding between ^{14}C-heptanal and soy proteins.

Modifying the protein by reacting with N-acetylimidazole increased the amount of ^{14}C-heptanal tightly bound to soy proteins, to a level well above the amount bound tightly to protein upon unfolding the protein with urea. Reaction with N-acetylimidazole makes the protein surface more hydrophobic by bonding to the tyrosyl residues of the protein. An increase in the hydrophobicity of the protein would encourage more hydrophobic bonding and thereby increase the amount of ^{14}C-heptanal bound to the protein via hydrophobic bonding. These data also support the fact that tight bonding of ^{14}C-heptanal to soy proteins were a result of hydrophobic bonding, and that chemical bonding with the tyrosyl groups was not a major contributor to this tight interaction.

Methodology and interpretation of data in studies of ligand binding to soy proteins

Several authors (Beyeler and Solms, 1974; Damodaran and Kinsella, 1980, 1981a b) have used equilibrium dialysis procedures to determine ligand binding to soy protein even with aldehyde and ketone ligands. These aliphatic aldehydes and ketones are known to react with the material used to build the dialysis apparatus which could cause an error in the analyses. This error could be constant or variable and thereby incorporate a significant error into the data obtained by these analyses. However, in these publications, no attempts have been reported in accounting for this error and for correcting it. Beyeler and Solms (1974) used ligand concentrations ranging from 10–80,000 ppm. The solubility of the ligands, used at the higher concentrations is questionable, for most medium chain length aliphatic aldehydes and ketones are only marginally soluble in aqueous mediums. Beyeler and Solms (1974), used a molecular weight of 50 kDa for soy proteins, which is incorrect. Furthermore, these studies were done using a whole soy protein sample, which was a mixture of proteins (including glycinin, β-conglycinin and other proteins).

According to Solms et al. (1973), soy proteins have compactly folded structures, while all the hydrophobic side chains were buried in the interior of the molecule. In contrast, Grinberg et al. (1989) have shown that glycinin has a non-compact structure. Solms et al., (1973) concluded that ligand binding to soy proteins was hydrophobic in nature and that it occurred with destruction of the tertiary structure so that the hydrophobic binding zones could be liberated from the interior of the molecule. However, Tanford (1973) has shown that proteins did have hydrophobic binding zones on the surface of the molecule, although most of the hydrophobic side

chains were buried in the interior of the molecule.

In an investigation conducted by Gremli (1974), on the interaction of some flavor compounds with soy proteins, it was concluded that alcohols did not bind to soy proteins. But according to Beyeler and Solms (1974), alcohols do bind to soy proteins. However, the soy protein used by Gremli (1974), was a denatured soy protein (Soyamin 90), and in that study, the protein had been separated from the supernatant by centrifuging at 200×g for only 10 min. In the present study, even at the isoelectric point of the purified proteins glycinin and β-conglycinin, centrifuging at 200×g for 10 min was not adequate to separate the protein from the supernatant. Unless this denatured soy protein (Soyamin 90) was extremely insoluble in an aqueous medium, this centrifugation would not have precipitated all the protein out of the solution. Moreover, since the ligand in the pellet and the supernatant were used as the bound ligand and the free ligand, respectively, this would have incorporated a significant error into these analyses.

Damodaran and Kinsella have investigated the binding of several aldehyde and ketone flavor compounds to crude soy proteins (1981a), and the binding of 2-nonanone to purified glycinin and β-conglycinin, separately (1981b). In both these studies, an equilibrium dialysis method was used to study the binding, while isooctane extraction was used to extract out the ligand from either side of the dialysis equilibrium set up. However, during the preliminary stages of the present study, attempts made to repeat this isooctane extraction of the ligand from the binding assay mixture proved unsuccessful. Even after extracting the binding assay mixture with hexane, isooctane and nonane (data not shown), there was ^{14}C-heptanal leftover in the binding assay mixture (as determined by radioactivity analyses). Furthermore, these solvents

formed emulsions with the binding assay mixture, which made it very difficult to separate the phases. A similar effect was observed by Kato et al. (1984) who studied the binding of SDS to several proteins including soy glycinin and β-conglycinin. These researchers were able to show that only free SDS could be extracted out of the SDS protein mixture, using $CHCl_3$ extraction. The bound SDS was not extracted into the $CHCl_3$ phase. Some of the observations and conclusions arrived at, during the investigations by Damodaran and Kinsella (1981ab), were conflicting. Even though the binding affinity changed with temperature, it was concluded that binding was hydrophobic in nature. Further, while their observations were that in the presence of urea, there was a reduction of binding of 2-nonanone to purified soy proteins, their conclusion still was that binding was hydrophobic in nature. Urea is known to unfold the protein polypeptide chain releasing hydrophobic binding zones, buried in the interior of the native protein structure, which would favor hydrophobic bonding. The results of the present study have shown that a suggestion made by Damodaran and Kinsella (1981b) for reducing the off-flavor of soy products by treating soy products with urea followed by dialysis, would not work. Treating soy products with urea, only increased the amount of aldehyde (^{14}C-heptanal) tightly bound to the soy proteins. Although these tightly bound heptanal molecules were not removable under the experimental conditions used, they may be released under a different set of conditions causing an off-flavor problem. A change in pH or temperature of the soy protein may be caused by incorporating soy proteins in an acidic food product (such as summer sausages) or by cooking the soy food product. Under such conditions the hydrophobicity of the protein may change and release some of the tightly bound off-flavor compounds.

O'Keefe et al. (1991b) studied the binding of hexanal to purified glycinin and β-conglycinin in 0.3 M Tris buffer at pH 8.0. In this study, the experimental conditions had been controlled very carefully. The ligand concentration in the headspace of the binding assay (free ligand) was used to determine the bound ligand concentration as the difference between the total ligand and the free ligand. Even though very dilute solutions (in the concentration range where Henry's law was applicable) were used, the interaction between the protein and the ligand had to be completely reversible for the ligand to behave as an ideal solution. If the presence of the protein caused the ligand to deviate from ideal solution behavior, then the basis for these analyses (Henry's law applicable to ideal or near ideal solutions) would not hold. The change in hydrophobicity upon ligand binding was studied using 1,8-anilino-napthalosulfonic acid (ANS) as a hydrophobic probe. The protein-bound form of the ANS is fluorescent and the interaction between ANS and proteins is hydrophobic in nature. A slight increase in hydrophobicity of the protein was observed as the level of hexanal equilibriated with the protein was increased. ANS being a hydrophobic ligand, would compete for the same binding sites as the hexanal bound to soy protein by hydrophobic interactions. Hence the use of ANS binding as a method of determining change in hydrophobicity of the protein upon ligand binding is questionable. O'Keefe et al. (1991a) reported that when using purified glycinin and β-conglycinin proteins, that saturation was not reached even at 1000 ppm ligand concentration with some aldehyde and ketone ligands, although saturation was reached with hexanal. A similar response was observed in the present study where saturation was not reached at 1000 ppm ^{14}C-heptanal with either of the soy proteins studied. The values of K reported by O'Keefe et al. (1991ab) for hexanal and octanal binding to glycinin were 270 ± 24

and 833 ± 76 M^{-1}, the corresponding values for β-conglycinin were 256 ± 160 and 200 ± 63 M^{-1}. The values of n reported by O'Keefe et al. (1991ab) for hexanal and octanal binding to glycinin were 96 ± 6 and 76 ± 6, the corresponding values for β-conglycinin were 32 ± 7 and 38 ± 5. Furthermore, their observations that equilibrium binding constants for glycinin increased as chain length increased for the aldehydes, and that $T|\Delta S| > |\Delta H|$, both support the involvement of hydrophobic interaction in aldehyde binding to soy proteins. However, these researchers had not arrived at a definite conclusion as to the type of bonds involved in aldehyde binding to purified soy proteins in an aqueous model system.

CONCLUSIONS AND RECOMMENDATIONS

A very selective and reasonably high yielding method of synthesis was developed for radiolabeled ^{14}C-heptanal. This method incorporated a ^{14}C-label and the selectivity of the procedure was such that the ^{14}C-heptanal was isolated in 90% purity. Although it was not possible to synthesize ^{14}C-labeled hexanal which is the main source of off–flavor in soy products, this method enabled the synthesis of a closely related compound ^{14}C-heptanal.

The synthesized ^{14}C-heptanal was used in studying the binding of this ligand to purified glycinin and β-conglycinin in an aqueous model system. The gel filtration technique used to monitor the binding of this ligand to soy proteins did not permit the determination of the total number of binding sites. The main reason for this failure was the limited solubility of the heptanal in the aqueous reaction assay solution used, which caused a phase separation before saturation was reached. However, these data indicate that glycinin and β-conglycinin had a higher binding capacity for ^{14}C-heptanal than that reached at 1000 ppm ^{14}C-heptanal concentration and that glycinin bound more heptanal per mol protein than β-conglycinin under the experimental conditions used. In this study, a large standard deviation was observed in the amount of ^{14}C-heptanal bound to protein and the amount of free ligand. However, under exhaustive elution conditions the standard deviations obtained for

the data on ligand binding were small and the data were very reproducible. This enabled the investigation of the presence of tightly bound heptanal on the soy proteins and the effect of protein modification on the ability to tightly bind ^{14}C-heptanal. As a result of these investigations, it was concluded that heptanal binding to soy proteins was not completely reversible due to the presence of tightly bound ligands. Moreover the data indicated that some of the heptanal bound to the soy proteins was weakly bound and easily removed, while another portion was tightly bound and difficult to be removed. This amount of tightly bound heptanal is a significant amount that could cause an off–flavor in a soy protein product if it was released. If we assume that hexanal behaved similarly to heptanal as far as interaction with soy proteins, then as an example, if a food product contained 1% soy protein in it, the amount of tightly bound aldehyde in this product would be 22.5 ppm aldehyde. Since the odor threshold for hexanal is 4.5 ppb, if even 1/1000 fraction of the tightly bound aldehyde was released from this product, then an off–flavor would be detectable.

The results of this study lead to the conclusion that the ^{14}C-heptanal tightly bound to glycinin and β-conglycinin, was bound by hydrophobic interactions. However, this tightly bound ^{14}C-heptanal does not account for all the heptanal bound to soy proteins. Electrostatic interactions may play a major role in the weaker binding of aldehyde to soy proteins. The nature of hydrophobic interactions is such that the interaction between the ligand and the protein is the weakest interaction, while the interaction between the ligand and the water molecules is moderate and the interaction between the water molecules is the strongest. As such, there is no chemical bond formation between ^{14}C-heptanal and glycinin or β-conglycinin. It is the attraction forces between water molecules that squeezes the hydrophobic ligands out of aque-

ous solution, causing them to aggregate with other hydrophobic molecules so that the contacts with water molecules are reduced. This means that it is the aqueous medium that causes ^{14}C-heptanal to bind tightly with glycinin and β-conglycinin. The proteins glycinin and β-conglycinin provide suitable hydrophobic surfaces either in the form of "hydrophobic patches" on the surface of the native protein or else in the form of hydrophobic zones in the unfolded polypeptide chain. The absence of chemical bond formation between the ligand and the protein, does not permit these hydrophobic ligands to be permanently bound to the protein. Thus, the association of these ligands with proteins would be determined by the hydrophobicity of the protein, the hydrophilicity of the solvent and the abundance of the ligand.

The hydrophobicity of the protein is predetermined by the genetic coding. This predetermined hydrophobicity was reported to change with pH and with heat treatment. According to Mills and Solms (1984) who studied the binding of heptanal to a partially denatured soy protein, more heptanal was irreversibly bound to the protein at pH 6.9 as compared to that at pH 4.7. These observations were made while using isooctane extraction of heptanal from the binding assay. Peng and Nielsen (1986) have shown that the surface hydrophobicity of β-conglycinin decreased upon heating to 50–100°C.

Several attempts have been made to reduce the abundance of hexanal and other off-flavor causing ligands by genetic breeding of low lipoxygenase containing varieties and by the use of antioxidants in soy foods. Low lipoxygenase varieties have not yet been developed into successful crop varieties. Even though these two procedures consist of a subset of conditions that would be required to solve the off-flavor problem in soy foods, these methods alone would not be adequate. As for changing

the hydrophobicity of the surrounding medium (solvent), there are not very many options available, as water is the most common liquid medium in soy foods for human consumption. Hence, changing the surface hydrophobicity of the protein would be an avenue with prospects for future research. It would be interesting to see whether a heat and pH change treatment could release all the tightly bound off–flavor compounds without destroying the protein functionality. Applying vacuum on the soy food is known to reduce the off–flavor in soy foods (Gremli, 1974; Arai et al., 1970a), probably by removing the loosely bound off–flavor compounds from the soy foods. Thus, if a pH and/or temperature treatment that could release the tightly bound off–flavor compounds from the soy proteins was available and a vacuum is applied to the soy food under those conditions, it may be possible to completely remove the off–flavor from the soy foods. Further, it would be interesting to study the effect of modifying the protein surface by making it more charged by bonding a multifunctional group like salicylic acid onto the protein surface. The charge introduced onto the protein surface by salicylic acid would make the protein surface less favorable for hydrophobic ligands to interact with.

In view of the world food shortage and the lack of adequate protein in the diet of some human beings, it would be beneficial to solve the off–flavor problem in soy foods, as soybeans contain high contents of valuable proteins. This would also enable the utilization of soy proteins, with excellent functional properties, to its greatest extent in substituting for more expensive meat proteins and also to be used in higher ratios in a greater variety of food products.

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