Autoacylation of Soy Proteins

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Soy proteins were autoacylated with palmitoyl-CoA. Soy proteins were denatured with 6 M urea and/or reduced with 0.2 M 2-mercaptoethanol, and then the reagents were removed by gel filtration. The reduced and the denatured and reduced glycinins were well acylated, although the native and the denatured glycinins were not. These observations demonstrated that cleavage of disulfide bonds was essential for the autoacylation. In addition, the autoacylation was strongly inhibited by the presence of urea and detergents. Inhibition of the autoacylation by 2-mercaptoethanol and *N*-ethylmaleimide and the release of palmitic acid by 2-mercaptoethanol and hydroxylamine indicated that palmitic acid was covalently linked via thioester bond to the cysteine residue. The autoacylation of glycinin was dependent on time, temperature, pH, and concentrations of palmitoyl-CoA and glycinin but independent of the saturation of fatty acids. β-Conglycinin was not acylated with palmitoyl-CoA.

Surface properties of proteins play one of the most important functions in food application (Kinsella et al., 1985). Fortification of the surface properties of food proteins increases their utilizability for food usage. The amphipathic nature of food proteins is responsible for their surface properties. Therefore, enhancement of the amphipathic nature of food proteins improves their surface properties. Covalent attachment of fatty acids to soy glycinin and α_{s1}-casein by a base-catalyzed ester-exchange reaction caused an increase in their surface properties (Haque and Kito, 1982, 1983a,b; Haque et al., 1982).

Acylated peptide and proteins are not new in nature. A large number of acylated proteins through covalent attachment of fatty acids have been reported (Stoffyn and Folch, 1971; Hantke and Braun, 1973; Schmidt et al., 1979; Omary and Trowbridge, 1981; Liau et al., 1986). The covalent attachment of the fatty acids occurs cotranslationally or posttranslationally by a specific acyltransferase (Schmidt and Schlesinger, 1980). Recently, the acylation of some proteins by an autocatalytic process has been reported (Berger and Schmidt, 1984; O'Brien et al., 1987; Bizzozero et al., 1987).

Soy proteins are important food materials from the viewpoints of economics and production, and they are comprised of two major components, glycinin and β-conglycinin. We describe here the autoacylation of soy proteins, in which glycinin is susceptible to autoacylation.

MATERIALS AND METHODS

Materials. Soy seeds (*Glycine max*, var. Tsuru-no-ko) were purchased from Mizuno Seed Co., Ltd. (Kyoto, Japan). Sephadex A-50 Fine was from Pharmacia Co., Ltd. (Uppsala, Sweden). [1-^{14}C]Palmitoyl-CoA and [1-^{14}C]oleoyl-CoA were from Du Pont/NEN Research Products (Boston, MA). Urea and 2-mercaptoethanol, extrapure reagent, were obtained from Nakalai Tesque, Inc. (Kyoto, Japan). Sodium dodecyl sulfate ($NaDodSO_4$), electrophoretic grade, was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of guaranteed reagent grades.

Preparation of Glycinin and β-Conglycinin. Acetone powder of a crude soy extract was prepared from dry seeds, as described by Mori et al. (1981). Crude glycinin and β-conglycinin fractions were prepared from acetone powder according to the method of Thanh and Shibasaki (1976). The crude fractions were dialyzed against distilled water (pH 7.5) and then lyophilized. The purities of these two protein fractions were >90% and >75%, respectively.

Pretreatment of Proteins. Lyophilized native proteins (glycinin or β-conglycinin, 10 mg) were dissolved in 25 mM Tris-HCl buffer (pH 7.5) containing 6 M urea and 0.2 M 2-mercaptoethanol (both denatured and reduced state), 6 M urea (denatured state), or 0.2 M 2-mercaptoethanol (reduced state). After being allowed to stand at 4 °C for 15 h, each sample was applied to a Sephadex G-50 Fine column (1.2 × 9 cm) equilibrated with 25 mM Tris-HCl buffer (pH 7.5) to exclude urea and/or 2-mercaptoethanol.

Incubation Conditions for Autoacylation. Native or pretreated proteins (250 μg) were incubated with 12 μM [1-^{14}C]-palmitoyl-CoA in 250 μL of 25 mM Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl. These are the standard conditions denoted in this paper. To stop the reaction, 1.2 mL of cold acetone was added and kept at −20 °C for 15 h. Finally, the suspension was centrifuged at 10000*g* for 5 min, and the pellet was processed for electrophoresis.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis ($NaDodSO_4$-PAGE). Labeled proteins were washed with cold acetone three times, dried in vacuo, and dissolved in 100 μL of $NaDodSO_4$ sample buffer (62.5 mM Tris-HCl buffer (pH 6.8) containing 3% SDS and 10% glycerol). The protein samples in $NaDodSO_4$ sample buffer were treated at 100 °C for 5 min, and then electrophoresed on $NaDodSO_4$ gel (11%) according to the method of Laemmli (1970). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 and destained. The bands of the constituents of glycinin and β-conglycinin were excised (1 cm × 2 mm) and solubilized by 0.5 mL of NCS tissue solubilizer (Amersham, U.K.) at 50 °C for 2 h in vials. After incubation, 17 μL of acetic acid was added to neutralize the solution, then 5 mL of Aquasol II (Du Pont/NEN) was added, and the radioactivity was measured on a Packard Tricarb 4530.

Release of Palmitic Acid from Palmitoyl Proteins. To elucidate the nature of the linkage of proteins and palmitoyl residue, the following experiment were carried out. The protein solution incubated with palmitoyl-CoA as described in the above section was mixed with 50–200 mM 2-mercaptoethanol and kept for 16 h at 25 °C. After incubation, cold acetone was added to the reaction mixture. The resultant pellet was analyzed by $NaDodSO_4$-PAGE as described above. Alternatively, the stained gels containing palmitoyl proteins were soaked in 1 M hydroxylamine (pH 10) or 1 M Tris-HCl buffer (pH 7.5 or 10) at 37 °C for 24 h. After incubation, the bands corresponding to the constituent polypeptides of glycinin were excised and then the radioactivity was measured as described above.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

RESULTS

Autoacylation of Soy Proteins. The autoacylation activity of soy proteins (glycinin, β-conglycinin) in native, denatured, reduced, and both denatured and reduced states (see Materials and Methods) is summarized in Table I. The reduced glycinin reacted well with palmitoyl-CoA, and both denatured and reduced glycinin exhibited twice as much reactivity as the reduced glycinin. The native and the denatured glycinins did not react with palmitoyl-CoA. These results indicate that a reduction of the disulfide

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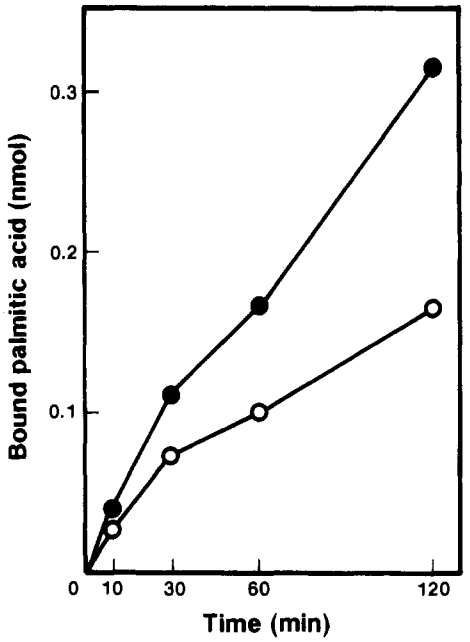

Figure 1. Time course of autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with polmitoyl-CoA at 37 °C for the indicated time as described in Materials and Methods. Key: (○) acidic polypeptide; (●) basic polypeptide.

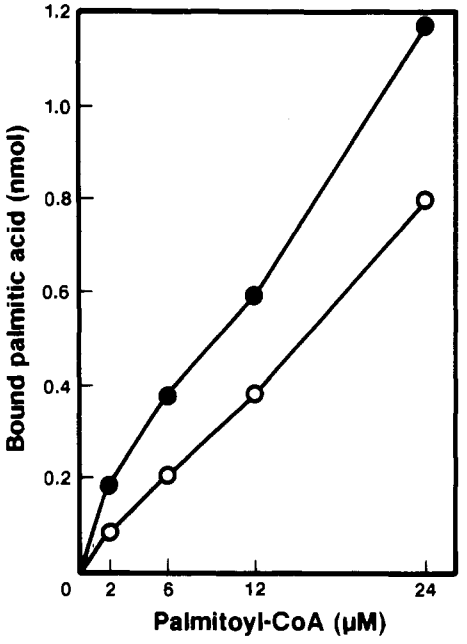

Figure 2. Effect of concentrations of palmitoyl-CoA on the autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with various concentrations of palmitoyl-CoA at 50 °C for 2 h as described in Materials and Methods. Symbols are the same as in Figure 1.

bonds of glycinin is essential for the reaction with palmitoyl-CoA. On the other hand, β-conglycinin did not react with palmitoyl-CoA even in the denatured and reduced state.

Characterization of Autoacylation of Glycinin. The extent of the autoacylation of the acidic and basic polypeptides of glycinin varied in proportion to the reaction time (Figure 1) and the concentrations of palmitoyl-CoA (Figure 2). The amount of palmitic acid incorporated into glycinin depended on the amount of protein (Figure 3). The concentration (0–450 mM) of NaCl had no effect on the autoacylation (data not shown). The autoacylation was

Table I. Autoacylation of Glycinin and β-Conglycinin

protein	palmitic acid incorporated, nmol			
	native	D[a]	R[a]	DR[a]
β-conglycinin	0.006			
α-subunit		ND[a]	ND	0.014
α′-subunit		ND	ND	0.015
β-subunit		ND	ND	0.026
glycinin	0.005			
acidic polypeptide		<0.005	0.163	0.396
basic polypeptide		<0.005	0.402	0.690

[a] Protein samples (250 μg) of native, denatured, reduced, and both denatured and reduced states were incubated with palmitoyl-CoA as described in Materials and Methods. After incubation, samples were analyzed by $NaDodSO_4$-PAGE and radioactivity was measured as described in Materials and Methods. Key: D and R, denatured and reduced, respectively; ND, not determined.

Table II. Effect of Various Reagents on the Autoacylation of Glycinin[a]

additive	autoacylation, %
none	100
6 M urea	27
0.15% Nonidet P-40	23
0.15% Triton X-100	21
0.15% Tween 20	32
0.15% $NaDodSO_4$	20
10 mM *N*-ethylmaleimide	5
100 mM *N*-ethylmaleimide	0.8

[a] Both reduced and denatured glycinin (250 μg) under the standard reaction conditions was mixed with each reagent indicated in the table and incubated for 1 h at 37 °C. Then, palmitoyl-CoA was added and the mixture was incubated at 50 °C for 2 h. After the reaction, samples were analyzed by $NaDodSO_4$-PAGE and the radioactivity was measured as described in Materials and Methods.

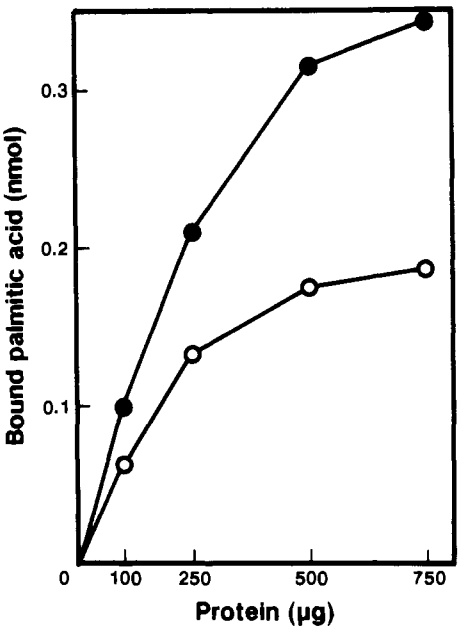

Figure 3. Effect of concentrations of glycinin on its autoacylation. Both denatured and reduced glycinin in different amounts were incubated with palmitoyl-CoA at 37 °C for 2 h as described in Materials and Methods. Symbols are the same as in Figure 1.

enhanced at a higher pH as shown in Figure 4 and increased with temperature up to 60–70 °C (Figure 5). The oleoyl residue of oleoyl-CoA was also incorporated into glycinin in a manner similar to that of palmitoyl-CoA (Figure 6), suggesting that the autoacylation proceeded regardless of whether the fatty acid was saturated or unsaturated. The autoacylation was 70–80% inhibited in the presence of urea and detergents (Table II).

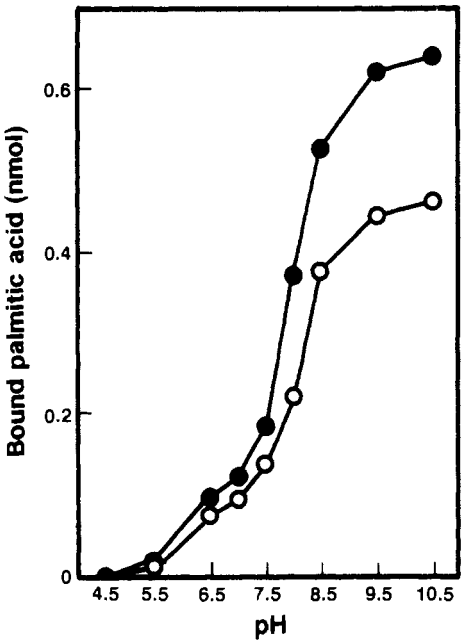

Figure 4. Effect of pH on the autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with palmitoyl-CoA at 37 °C for 2 h under various pH. Citrate–phosphate buffer (25 mM, pH 4.5–5.5) and Tris-HCl buffer (25 mM, pH 6.5–10.5) were used. Symbols are the same as in Figure 1.

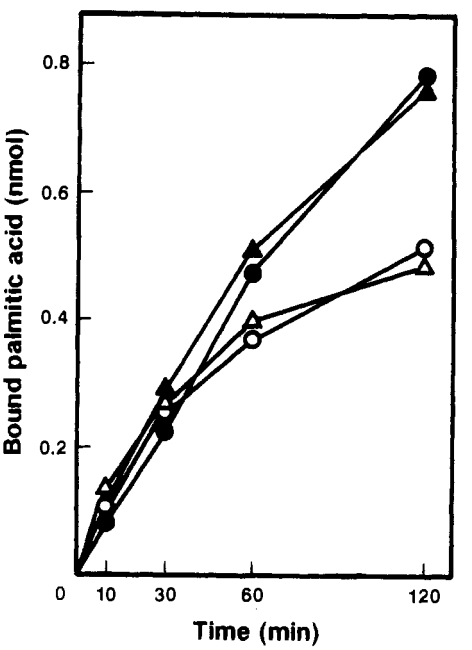

Figure 6. Effect of acyl residues on the autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with palmitoyl-CoA (circle) or oleoyl-CoA (triangle) at 50 °C for 2 h. Open and black symbols are acidic and basic polypeptides, respectively.

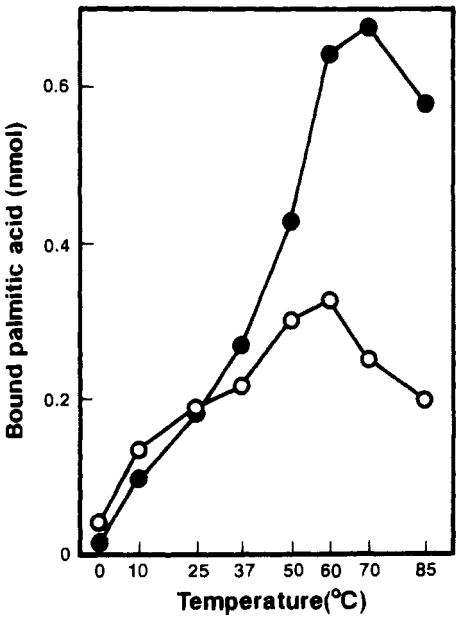

Figure 5. Effect of temperature on the autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with palmitoyl-CoA for 2 h at various temperatures. Symbols are the same as in Figure 1.

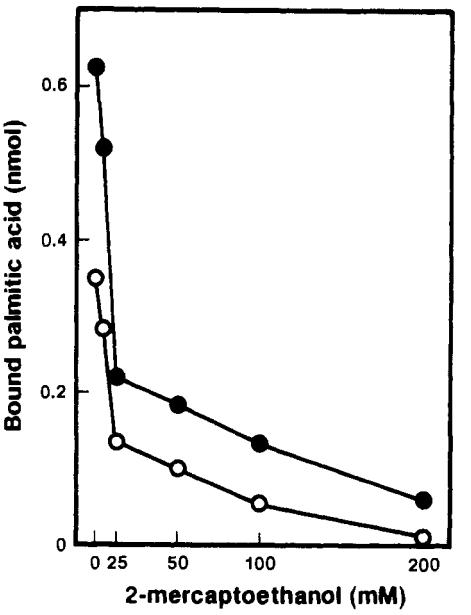

Figure 7. Effect of 2-mercaptoethanol on the autoacylation of glycinin. Both denatured and reduced glycinin (250 μg) were incubated with palmitoyl-CoA at 50 °C for 2 h in the presence of various concentrations of 2-mercaptoethanol. Symbols are the same as in Figure 1.

Site and Type of Incorporation. The necessity of the reduction of disulfide bonds for the autoacylation of glycinin suggests that cysteine residue is required for the acylation. To confirm this, the following experiments were examined. The presence of 2-mercaptoethanol in the reaction mixture resulted in 60% inhibition of the autoacylation at 25 mM and >90% inhibition at 200 mM (Figure 7). The blocking of SH groups with *N*-ethylmaleimide supressed the autoacylation almost completely (Table II). Hence, it appears likely that cysteine residue interacts covalently with the palmitoyl residue.

In order to see the type of linkage between the cysteine residue and the palmitoyl residue, the stability of the linkage was examined. It is known that ester or thioester linkages to the side chains of serine, threonine, and cysteine residues are sensitive to hydroxylamine, but amide linkages are not (Kaufman et al., 1984). The acylated glycinins separated by $NaDodSO_4$ gels were soaked in 1 M hydroxylamine (pH 10) at 37 °C. After 24 h, 65% of the incorporated palmitic acid was released. The amount was higher than that in 1 M Tris-HCl (pH 10) (Table III). When acylated glycinin was incubated with 2-mercaptoethanol at 25 °C, 40–50% of the incorporated palmitic acid was released after 16 h at concentrations above 50 mM 2-mercaptoethanol (Figure 8). These results indicate that palmitic acid may be linked via thioester bonds to cysteine

Table III. Release of Bound Palmitic Acid from Glycinin[a]

treatment	release of [^{14}C]palmitic acid, %
1 M Tris-HCl, pH 7.5	0
1 M Tris-HCl, pH 10	31
1 M hydroxylamine, pH 10	65

[a] NaDodSO$_4$–polyacrylamide gels containing acylated glycinin were soaked in each solution indicated in the table and incubated at 37 °C for 24 h as described in Materials and Methods. After incubation, the bands corresponding to glycinin were cut out and radioactivity was measured as described in Materials and Methods.

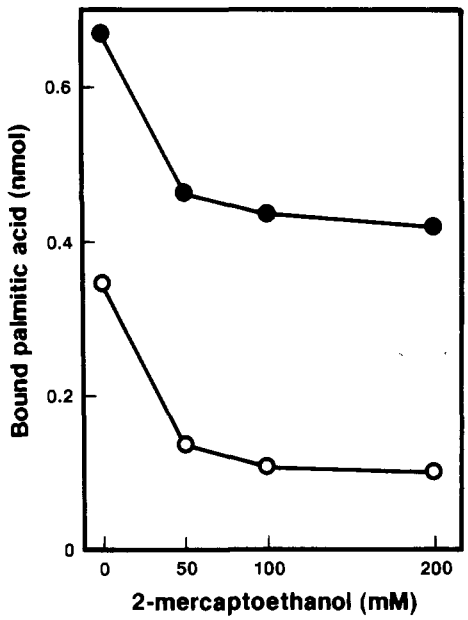

Figure 8. Release of palmitic acid from acylated glycinin by 2-mercaptoethanol. Both denatured and reduced glycinin were incubated with palmitoyl-CoA at 50 °C for 1 h as described in Materials and Methods. After the reaction, various concentrations of 2-mercaptoethanol were added and the reaction mixture was incubated for 16 h at 25 °C. Symbols are the same as in Figure 1.

residues of the acidic and basic polypeptides of glycinin.

DISCUSSION

There is a large number of proteins to which fatty acids are covalently bound in nature. Such an acylation is catalyzed by a protein acyltransferase, which exists in microsomal membranes (Berger and Schmidt, 1984). Recently, autoacylation of semliki forest viral polypeptides (Berger and Schmidt, 1984), disc membrane rhodopsin (O'Brien et al., 1987), and myelin proteolipid protein (Bizzozero et al., 1987) have been reported. Native glycinin did not react with palmitoyl-CoA. The addition of microsomal membrane fractions prepared from rat liver to the reaction mixture did not exhibit any effects on acylation (data not shown). On the other hand, the reduced glycinin was acylated with palmitoyl-CoA but the denatured glycinin was not. These observations indicate that the acylation certainly proceeded by an autocatalytic process, but not by the catalytic activity of glycinin itself. The reduction of disulfide bonds is essential for the autoacylation. It is not excluded that a protein acyltransferase that is either resistant to denaturation or refolds to restore activity upon renaturation exists in the glycinin preparation.

Glycinin that was denatured and reduced exhibited twice as much reactivity as the reduced glycinin. The addition of urea and detergents to the reaction mixture resulted in 70–80% inhibition. Therefore, it is likely that some conformation that is favorable for the autoacylation may be induced by restricted renaturation of the denatured and reduced glycinin during gel filtration. This was supported by the finding that the reactivity of both denatured and reduced glycinin decreased to about 40% with dialysis against 25 mM Tris-HCl buffer (pH 7.5) for 18 h, instead of gel filtration. During dialysis considerable renaturation occurred. These phenomena are different from that of myelin proteolipid protein, which needs its native structure (Bizzozero et al., 1987). There is a possibility that urea and detergents disrupt heterointermolecular hydrophobic association.

The fatty acids were linked via thioester bonds to the cysteine residues of glycinin. This is similar to the case of rhodopsin (O'Brien et al., 1987), but not that of myelin phospholipid protein, *O*-ester bonds (Bizzozero et al., 1987). Draper and Catsimpoolas (1978) demonstrated that glycinin contains two free sulfhydryl groups and twenty disulfide bonds per mole of protein. Only a small portion of the two free sulfhydryl groups is reactive with DTNB in the native state, indicating that most of the free sulfhydryl groups are located internally (Nakamura et al., 1984). This may be one of the reasons why the native glycinin exhibited little reactivity. The differences in the reactivity among the states of glycinin are likely due to the availability of free sulfhydryl groups, in addition to the changes in protein conformation discussed above. Under any set of reaction conditions, the basic polypeptides exhibited higher reactivity with palmitoyl-CoA than the acidic polypeptides. The acidic polypeptides contain more half-cystines than the basic polypeptides (Nielsen, 1985). Therefore, the difference in reactivities between the acidic and the basic polypeptides may be due to the environmental differences around the cysteine residues, such as hydrophobicity and hydrophilicity. β-Conglycinin did not react with palmitoyl-CoA. This may be due to the fact that β-conglycinin contains a small number of cysteine residues (Thanh and Shibasaki, 1978; Nielsen, 1985; Doyle et al., 1986).

In the case of rhodopsin and myelin phospholipid protein, acylation proceeds at a higher rate with palmitoyl-CoA than with myristoyl-CoA, indicating that substrate specificity was maintained. However, glycinin exhibited no substrate specificity. The autoacylations of glycinin, myelin phospholipid protein, and rhodopsin have some features in common with each other: The acylations are time and temperature dependent (Bizzozero et al., 1987; O'Brien et al., 1987).

Emulsification activity and surface hydrophobicity of an acylated glycinin into which ≈0.06 mol of palmitic acid was incorporated were measured by the methods of Pearce and Kinsella (1978) and Kato and Nakai (1980), respectively. Emulsification activity increased to 160% and surface hydrophobicity to 127%, compared with the native glycinin. The amount of palmitic acid incorporated into the glycinin subunit is 0.3 mol/mol of glycinin subunit (about 5% of the average number of cysteine residues per subunit) under optimum conditions. However, we are trying to increase the amount of palmitic acid incorporated into glycinin. We will then be able to create an acylated glycinin with much better surface properties than those of the native glycinin.

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Chlorogenic Acid Interactions with Sunflower Proteins

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The interaction of chlorogenic acid with sunflower (*Helianthus annuus*) protein isolate was investigated with continuous diafiltration at three ligand to protein molar ratios and at four pHs. The pH had a profound influence on the extent of binding. The molar binding ratio was lowest at pH 5, irrespective of ligand to protein molar ratio. Binding was greater at pH 7 and pH 3. At pH 9, binding was lower than at pH 3 up to a certain free chlorogenic acid concentration. Above this concentration, binding at pH 9 was higher than at other pHs. Binding increased as the ligand to protein molar ratio increased irrespective of pH. There are two groups of binding sites in sunflower proteins at pH 3, 5, and 7 and three groups at pH 9.

Polyphenolic compounds have been a major deterrent to large-scale use of sunflower proteins in food products, due to their chromophoric properties. If the pH of sunflower flour or meal, which contains 3–5% by weight phenolic compounds, is raised above neutrality, its color progresses from a cream yellow to light green, to dark green, and finally to brown. It is desirable for vegetable proteins to be odorless, bland, and colorless. Many of the current methods for producing such protein isolates either mask the color changes or add extra processing steps that increase the cost. Understanding the reaction mechanisms can lead to innovative processes to develop such products.

Phenolic compounds in sunflower products include chlorogenic acid, caffeic acid, and quinic acid (Joubert, 1955; Sechet–Sirat et al., 1959), varying with the location of the seed on the sunflower head, storage temperature (Pomenta and Burns, 1971), and variety (Sosulki et al., 1972). Chlorogenic acid (3-*O*-caffeoyl-D-quinic acid) is the major phenolic compound. Acid, base, or enzymatic hydrolysis of the ester linkage of chlorogenic acid yields caffeic acid and D-quinic acid. True chlorogenic acid is

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