Incorporation of Fatty Acid into Food Protein: Palmitoyl Soybean Glycinin

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Palmitic acyl residue was incorporated into soybean glycinin in the presence of 8 M urea at pH 9.0, 25 °C, with and without 2-mercaptoethanol. *N*-Hydroxysuccinimide ester of palmitic acid was used as the lipophilic electrophile. The palmitoyl proteins showed higher emulsifying activity and foam stability than the standard glycinin protein. The sedimentation coefficients of these palmitoyl proteins varied between 3 and 4 S. Thin-layer chromatographic analysis of Pronase-E hydrolyzed palmitoyl peptides showed extractability into a apolar solvent system of the hydrophobic anchor along with four to five amino acid residues. Enzymatic hydrolysis employing an in vitro model system indicated decreased hydrolyzability of the palmitoyl proteins.

The importance of improving the functional properties of food proteins have greatly been stressed upon in recent years. Nonanimal proteins, due to their potentially larger supply and lower cost, especially soybean proteins, have generated particular interest (Kinsella, 1979; Wolf and Cowan, 1975). However, limited improvement in the functional properties and adverse effect on the organoleptic qualities have been the major factors preventing increased utilization of these and other novel proteins (Kinsella, 1976; Rackis, 1979).

Chemical modification of food proteins under mild conditions should enhance functional properties without detrimental effect on their nutritional value (Feeney, 1977b). Chemical derivatization of the lysine residues by acylation with succinic anhydride has been suggested as a means of enhancing food protein functionality (Kinsella and Shetty, 1978). Tyrosyl ester and thioester are also formed, but these are sponteneously hydrolyzed in the aqueous media within a few hours (Riordan and Vallee, 1964; Gounaris and Perlmann, 1967). A number of food proteins have thus been treated with varying degrees of success. Other reagents that have been used for the chemical modification of food proteins have been extensively reviewed by a number of authors, including Glazer (1976) and Feeney (1977a).

The development of our understanding of membrane structure and its importance in the biological system has led to a renewed interest in the study of protein–lipid interaction. These interactions have long been recognized to play critical roles in determining the functional properties of many food proteins (Karel, 1973; Pomeranz, 1973; Webb et al., 1974). An example of the role of protein–lipid interactions in functionality which illustrates both their importance and complexity is the role of protein–lipid interaction in maintaining the structure of wheat-flour dough. Tsen et al. (1971) reported the usefulness of certain polar lipids in improving the functional quality of baked wheat-flour products that have been nutritionally supplemented with soy flour. In a related piece of work, Hoseney et al. (1970) reported that polar lipids of dough bind to the gliadin proteins largely by hydrophilic bonds and simultaneously to glutenin proteins by largely hydrophobic bonds.

Kito and colleagues (Ohtsuru et al., 1976; Kanamoto et al., 1977; Kito et al., 1979) reported the association of phosphatidylcholine with soybean proteins being due to the hydrophobic interaction between the phosphatidylcholine molecule and the hydrophobic region of the proteins and also due to the binding of phosphatidylcholine lamellae to the protein surface. Covalent attachements have not been reported.

It would be interesting to observe the protein functional changes brought about by the covalent anchoring of hydrophobic moieties onto a hydrophilic protein molecule and thereby enhancing its surface active function. Belikow et al. (1975) have reported the covalent attachment of hydrophobic ligands to casein. They, however, did not provide direct evidence regarding the degree of incorporation. Arai and Watanabe (1980) have reported the enzymatically induced covalent attachment of L-norleucine dodecyl ester to succinylated α_{S1}-casein resulting in one such bonding at the C-terminal position of the hydrophilic substrate molecule. However, casein was fragmented by the enzyme prior to the incorporation.

Our present work involves the incorporation of a lipophilic component into the hydrophilic soybean glycinin modecule by base-catalyzed ester exchange. The *N*-hydroxysuccinimide ester of palmitic acid was used as the lipophilic electrophile.

EXPERIMENTAL SECTION

Materials. Freshly harvested soybean seeds (*Glycine max.*, var. Tsuru-no-ko) (1980) of Hokkaido prefecture were obtained from Takii Seed and Seedling Co., Kyoto. Pepsin (1:60 000), aminopeptidase (microsomal type VI) (EC.3.4.11.2), prolidase (EC.3.4.13.9), and pancreatin were purchased from Sigma Chemical Co., St. Louis, MO. Pronase-E (1:1 000 000) was purchased from Kaken Chemical Co., Tokyo. TLC plates (Art. 5721) with a layer thickness of 0.25 mm were obtained from Merck, Darmstadt, West Germany. CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The ultrafiltration membrane (PM-10) was from Amicon, Lexington, MA, and filter type HA of pore size 0.45 μm was from Millipore Corp., Bedford, MA. Palmitic acid (16:0), 2-mercaptoethanol (2-ME), deionized urea, *N*-hydroxysuccinimide (HOsu), and dicyclohexylcarbodiimide were obtained from Nakarai Chemicals, Ltd., Kyoto. All other reagents were of analytical grade.

Methods. *Preparation of Soybean Glycinin.* Defatted soybean acetone powder was prepared according to the method of Ohtsuru et al. (1976), and glycinin was then prepared as described by Thanh and Shibasaki (1976). The protein thus obtained was stored for short periods in 2% concentration at −20 °C in the 0.5 μ standard buffer and thawed and centrifuged at 25 °C just prior to use. Protein concentrations were routinely determined by the relationship $E_{280}^{1\%,1cm} = 8.04$ (Koshiyama, 1972).

Buffer. Potassium phosphate buffer, 33 mM, pH 7.6, containing 10 mM 2-ME and 0.4 M NaCl (ionic strength

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0.5) (Wolf and Briggs, 1959) was used as the 0.5 μ standard buffer. Modifications when required have been indicated in the text.

Esterification of Palmitic Acid with N-Hydroxysuccinimide. Palmitic acid, 31.5 mmol (8.1 g), and *N*-hydroxysuccinimide, 31.5 mmol (3.6 g), were dissolved in 62.5 mL of tetrahydrofuran (THF), and the temperature was equilibrated to 0 °C in a ice bath. Dicyclohexylcarbodiimide, 31.5 mmol (6.5 g), was then gradually added while stirring over a period of 30 min following which the mixture was stirred at 0 °C for 2 h and at 25 °C overnight. The dicyclohexylurea thus formed was then vacuum filtered, and the filtrate was evaporated to be redissolved in ethyl acetate (100 mL). The solution was then washed with 10% NaCl and water, filtered through a phase separator filter paper (Whatman 1 PS), dried over Na_2SO_4, and then evaporated under reduced pressure. The product was recrystallized from ethyl acetate–petroleum ether. The yield of the white flaky *N*-hydroxysuccinimide ester of palmitic acid (16:0-Osu) was 66%, and its purity was confirmed by thin-layer chromatographic analysis using the solvent system of petroleum ether–diethyl ether–acetic acid (80:20:1: v/v).

Preparation of Palmitoyl Protein. (1) Method A. Fifty milliliters of THF containing 625 mg of 16:0-Osu was added gradually to a 0.5% solution (450 mL) of glycinin in the 0.5 μ standard buffer containing 8 M urea, and the mixture was incubated at pH 9.0, 25 °C, for different time intervals. During the incubation the vessel was agitated at 50 strokes (225 mm) per min by using a "swing arm agitator". The reaction was then stopped by dialysis at 4 °C against the standard buffer containing 1 M NaCl. The protein solution was then washed 5 times with chilled (4 °C) diethyl ether, alternately centrifuged (2500 rpm; 0 °C; 5 min) to break the resulting emulsion, and dialyzed exhaustively for 72 h against two daily changes of deionized water (neutral pH) containing 10 mM 2-ME and a final change of distilled water (neutral pH) containing 10 mM 2-ME. The palmitoyl protein thus obtained was stored at 4 °C with 0.025% NaN_3. Different concentrations for the various experiments were achieved by ultrafiltration using a Amicon cell (Model 52) fitted with a PM-10 membrane.

(2) *Method B.* Method B was identical with method A except that 2-ME was excluded from all stages. Glycinin was made free of 2-ME used during storage by dialysis against the 0.5 μ standard buffer without 2-ME.

Preparation of Standard Glycinin. Soybean glycinin at 0.5% concentration in the 0.5 μ standard buffer was subjected to the above-mentioned dialysis steps only.

Determination of Degree of Incorporation. The incorporated 16:0 was determined by gas–liquid chromatography (GLC). A Shimadzu GC-7A fitted with a glass column packed with 10% Silar 10C on Chromosorb W, mesh 100–120, treatment AW-DMCS, was used within a temperature range of 160–240 °C. The injection temperature and program progress rate were 260 and 4 °C, respectively. Total 16:0 was determined after acid hydrolysis of the palmitoyl protein, followed by extraction with diethyl ether and methylation with diazomethane for 20 min at 25 °C. Free 16:0, if any, was determined without acid hydrolysis.

Amino Acid Analysis. A Hitachi KLA-5 amino acid analyzer was used for the quantitative estimation of amino acids in the protein hydrolysates by the sodium citrate buffer method of Spackman et al. (1958).

Enzymatic Hydrolysis. An in vitro model system of the gastric and postgastric protein hydrolysis was set up as described by Matoba et al. (1982). The system was a two-tier process first involving (1) pepsin–pancreatin followed by (2) aminopeptidase–prolidase hydrolysis.

(1) The protein sample (20 mg) was incubated in 3 mL of 0.1 N HCl with 0.3 mg of pepsin for 3 h at 37 °C while shaking. After neutralization, 1.5 mL of pancreatin solution was added and the mixture incubated for another 20 h at 37 °C. The pancreatin solution was the supernatent resulting from the centrifugation of the enzyme (50 mg) dissolved in 75 mL of 0.2 M 3-(*N*-morpholino)propanesulfonic acid containing 0.025% NaN_3 at pH 8.0, 0 °C. The hydrolysis was stopped by boiling for 5 min.

(2) A portion (0.5 mL, i.e., 6.66 mg) of the enzymatic digest was injected into a cartridge containing aminopeptidase coupled to Sepharose and prolidase coupled to Sepharose, each being 0.8 mL of the wet gel. The content of each enzyme per milliliter of wet gel was about 2.5 mg. The buffer used was 0.1 M borate, pH 8.0, containing 20 mM $MnCl_2$, 0.025% NaN_3, and 0.03% toluene. The hydrolysis was carried out for 24 h at 37 °C while shaking gently following which the hydrolysate was separated from the immobilized enzyme mixture by vacuum filtration.

Acid Hydrolysis. Proteins and peptides were hydrolyzed with redistilled 6 N HCl at 110 °C for 24 h in evacuated tubes in the presence of 15–20 μL of phenol for amino acid analysis and combined amino acid and 16:0 analysis. For 16:0 analysis alone, the hydrolysis was in Teflon-stoppered tubes for 4 h.

Thin-Layer Chromatographic Analysis of Palmitoyl Peptides. A 1% solution of the protein (100 mg) in 10 mL of the 0.5 μ standard buffer without 2-ME was hydrolyzed at pH 7.0, 40 °C, for 3 h with Pronase-E (5 mg). The pH was then adjusted to 6.0 with 0.1 N HCl and the solution extracted with the solvent chloroform–methanol–1-butanol (2:1:1 v/v/v). The extract was then concentrated and applied on a TLC plate and developed along with parallel references with chloroform–methanol–acetic acid (80:20:1 v/v/v). Appropriate sections were then scraped off and acid hydrolyzed followed by *n*-hexane extraction for GLC determination of the 16:0 content and centrifugation at 3000 rpm, 20 °C, for 5 min. The supernatants were evaporated at 40 °C under reduced pressure and redissolved in 0.2 M citrate buffer containing Brij and thiodiglycol and passed through a Millipore filter (type HA; pore size 0.45 μm) to remove residual TLC scraping before amino acid analysis.

Ultracentrifugal Analysis. Sedimentation analysis was performed with a Hitachi UCA-1 centrifuge at 51 200 rpm, 20 °C, in the 0.5 μ standard buffer to obtain the $s_{20,w}$ value. Schlieren optics were used.

Whipping Properties. Foam activity (FA) and foam stability (FS) were taken as the indices of the whipping property of proteins. If L^0 and L^1 are the volumes of the protein solution before and after shaking, and if F^1 and F^2 are the volumes of the foam after shaking and after standing for 30 min, respectively, FA and FS can then be expressed as

$$\mathrm{FA} = \left(\frac{L^1 + F^1}{L^0} - 1\right) \times 100 \quad (1)$$

$$\mathrm{FS} = \frac{F^2}{F^1} \times 100 \quad (2)$$

Stoppered graduated cylinders of 10-mL capacity were used for test volumes of 4 mL of 1% protein in the 0.5 μ standard buffer without 2-ME. A swing arm agitator was used at 120 strokes (110 mm) per min for 1 min at 25 °C for FA determination. FS was determined after standing

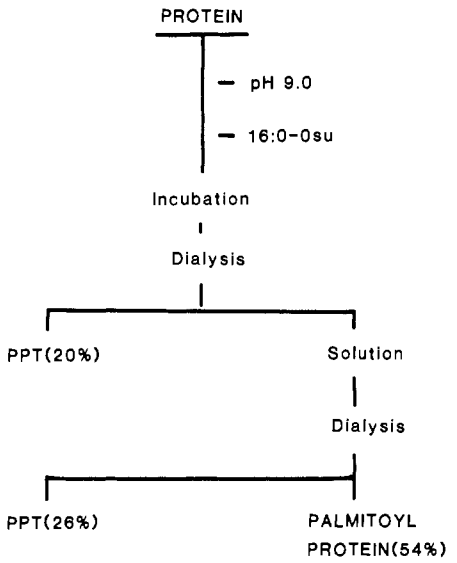

Figure 1. Preparation of palmitoyl protein.

for 30 min at the same temperature.

Emulsification Activity. The emulsification activity of the palmitoyl proteins was compared with that of the standard glycinin. As defined by Miller and Groninger (1976), the emulsification activity indicates whether a protein has the ability to emulsify and, if it does, the degree of emulsification for a given amount of the protein. The turbimetric method of end point determination as described by Pearce and Kinsella (1978) was used for our purpose.

The standard glycinin was emulsified at five concentrations between 0.4 and 2.0%; the palmitoyl proteins were emulsified at three concentrations between 0.4 and 1.2%.

The protein solution (10 mL) in 0.1 M sodium phosphate buffer, pH 7.0, was added to 2 mL of soybean oil placed in a small beaker of 15-mL capacity and was stirred at an uniform rate for 2 min at 25 °C while being simultaneously sonicated in a "horn-type" adapter fitted to a Branson sonifier (cell disrupter 200; 20 kHz) operated at maximum power. A 10-μL portion of the resulting emulsion was then pipetted off from the bottom to be placed in a test tube containing 10 mL of 1% (w/v) sodium dodecyl sulfate in water, and the tube was inverted 3 times to afford a homogeneous mixture following which the absorbance at 600 nm was immidiately read by using a Carl Zeis (M4 0III) spectrophotometer.

RESULTS

Synthesis of Palmitoyl Proteins. The covalent incorporation of 16:0 into glycinin at pH 9.0, 25 °C, was time dependent, influenced by the degree of mechanical agitation, and sensitive to pH alterations (data not shown). The incorporation carried out in the presence of 2-ME (method A) appeared to be higher than that carried out in its absence (method B). Different degrees of incorporation were obtained by adjusting the incubation time. After 30-min reaction, glycinin covalently incorporated with 11 mol of 16:0/mol of glycinin by method A and 5 mol of 16:0/mol of glycinin by method B was respectively called "palmitoyl protein A" and "palmitoyl protein B" and was considered for further study. The respective yields of palmitoyl proteins A and B were 54 and 77%. Figure 1 shows the stages at which the precipitations occurred for palmitoyl protein A.

TLC of Pronase-E Hydrolysate. The mobility of the amino acids in the developing solvent used (chloroform–methanol–acetic acid, 80:20:1 v/v), as seen in Figure 2, confirms their covalent attachment to 16:0. Trace amounts

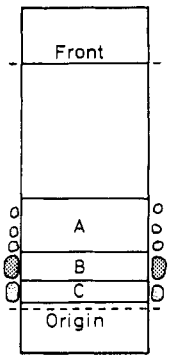

Figure 2. TLC development of Pronase-E hydrolysate of palmitoyl proteins. Parallel references were visualized with ninhydrin, and appropriate sections A, B, and C were determined and scraped off for acid hydrolysis.

Table I. Occurrence of Palmitoyl Peptides in Pronase-E Hydrolyzed Palmitoyl Protein A

amino acids, nmol/100 mg	TLC scrapings as shown in Figure 2			
	A	B	C	total
lysine	92	76	195	363
histidine		4	18	22
arginine	114	19	38	171
aspartic acid[a]	3	58	193	304
threonine	43	33	62	138
serine	29	33	84	146
glutamic acid[a]	47	44	133	124
glycine	138	70	76	286
tyrosine	32	33	61	126
alanine	38	38	90	166
valine	233	65	64	362
methionine	13	36	24	73
isoleucine	203	129	46	378
leucine	76	339	91	506
phenylalanine	101	327	58	486
total 16:0	226	160	339	725
amino acid to 16:0 ratio	4.9:1	8.1:1	3.6:1	5.2:1

[a] Asp and Glu also contain Asn and Gln, respectively.

of 16:0 and 16:0-Osu traveled to the solvent front. Pronase-E is apparently unable to hydrolyze the peptide bonds adjacent to the palmitoyl amino acid residues as is apparent from the amino acid to 16:0 ratio which was 5.2:1 and 4.2:1 for palmitoyl proteins A and B, respectively. Table I shows the amino acid content of the different TLC plate sections (Figure 2) in comparison to the total 16:0 recovered from palmitoyl protein A. Palmitoyl protein B (data not shown) showed a similar pattern, indicating similar hydrolysis by Pronase-E.

In Vitro Digestibility. In vitro digestibility tests were conduced to estimate the enzymatic hydrolyzability of palmitoyl protein A and compare this with that of the standard glycinin. Chemical hydrolysis was carried out side by side for comparison. The protein solution which was slightly opaque became clear within 10 min of pepsin–pancreatin digestion. However, the final enzymatic release of almost all the amino acids analyzed was a little lower for the palmitoyl protein. Table II shows the amino acids released by enzymatic and chemical hydrolysis of palmitoyl protein A compared to that for the standard glycinin. It should, however, be noted that the sodium citrate buffer system causes the peaks of glutamine and asparagine to overlap threonine and serine on enzymatic hydrolysis and those of glutamic acid and aspartic acid on acid hydrolysis.

Sedimentation Analysis. The ultracentrifugal analyses were all done at 1% concentration in the 0.5 μ standard

Table II. Enzymatic and Chemical Hydrolysis of Glycinin and Palmitoyl Protein A

free amino acids,[a] μmol/μmol of protein	enzymatic hydrolysate		chemical hydrolysate	
	palmitoyl protein	glycinin	palmitoyl protein	glycinin
lysine	121	133	124	123
histidine	42	49	55	54
arginine	139	140	168	167
aspartic acid	63	76	297	304
threonine	273	289	97	101
serine	244	262	158	165
glutamic acid	164	180	523	520
glycine	172	181	198	198
alanine	117	141	124	139
valine	130	143	122	134
methionine	28	30	29	30
isoleucine	116	122	111	116
leucine	172	189	162	189
tyrosine	66	60	68	67
phenylalanine	100	104	100	103

[a] For the enzymatic hydrolysate, Thr and Ser also contain Gln and Asn, respectively. Asp and Glu of the acid hydrolysate also contain Asn and Gln, respectively.

Table III. Functional Properties[a]

sample	foam act. (FA)	foam stability[b] (FS)	rel[c] emulsifying act.
glycinin	101	60	100
palmitoyl protein A	112	60	246
palmitoyl protein B	128	96	266

[a] Mean of three experiments. [b] The maximum calculated stability after 30 min is 100. [c] 0.4% protein solution in 0.1 M sodium phosphate buffer, pH 7.0, at 25 °C.

buffer. The sedimentation coefficients thus obtained for palmitoyl proteins A and B were between 3 and 4 S. Figure 3 shows the schlieren peak of the slow-sedimenting palmitoyl protein A in comparison with that of the faster sedimenting soybean glycinin (11 S) at 48 min after attaining speed.

Foam Activity and Stability. Foam activity and stability are shown in Table III. Palmitoyl protein A shows little difference in comparison with the standard glycinin. Palmitoyl protein B, however, showed significantly higher FS and a slightly higher FA.

Emulsifying Activity. The comparative emulsifying activity, as seen in Table III, shows that the palmitoyl proteins have significantly higher emulsifying activity. The results shown are those for 0.4% protein concentration. The oil to water ratio was 1:5 in all cases. At higher protein concentrations, it was observed that the amount of oil present was the limiting factor for the palmitoyl proteins contrary to the standard glycinin in which case the protein concentration was more important. The palmitoyl proteins showed almost similar emulsifying activity which was 246% that of standard glycinin for A and 266% that of standard glycinin for B.

DISCUSSION

Under the reaction condition of method A, glycinin was dissociated completely to the subunits (Kitamura and Shibasaki, 1975) which could not reassociate during the dialysis steps since disulfide bonding was prevented by the presence of 2-ME. The basic subunit therefore must have precipitated almost completely, leaving a soluble fraction, referred to as palmitoyl protein A, which may have consisted almost solely of incorporated acidic subunits. On the other hand, in method B the disulfide bridge connecting the acidic and basic subunit to form the intermediary subunit was not reduced as 2-ME was not added at any stage. The protein thus derived and referred to as palmitoyl protein B may therefore have been mostly composed of incorporated intermediary (acidic–basic) subunits. The similar sedimentation behavior of palmitoyl proteins A and B (Figure 3) may be because the acidic subunits of the former existed as oligomers (acidic–acidic) (Yamagishi et al., 1980) due to hydrophobic interaction of the hydrophobic tails.

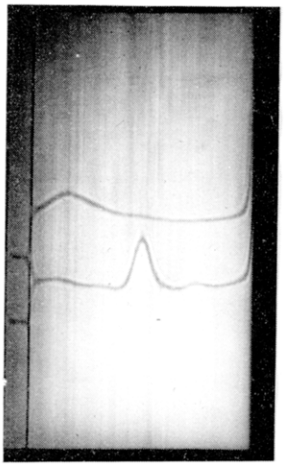

Figure 3. Ultracentrifugal schlieren peak of palmitoyl protein A. The upper peak is that of palmitoyl protein A. The lower peak is that of soybean glycinin. Sedimentation is from the left to the right.

The primary objective of the first dialysis against the standard buffer containing 1 M NaCl (with and without 2-ME) was to quench the reaction and to increase the salt concentration which reduced emulsification and thus promoted proper cleansing of the palmitoyl protein solution during the subsequent washing step. Initial experiments indicated that free 16:0 and 16:0-Osu could not be eradicated unless the dialysis step was introduced prior to the washing step. The other objectives of the first dialysis were to reduce the solution temperature so as to avoid denaturation during the washing step and to screen out (1) the basic subunits (method A) and (2) excessively denatured protein (method B). It may be noted that in the absence of 2-ME the buffer used had a reassociating effect (Wolf and Briggs, 1958). The second dialysis against deionized water at around neutral pH was essential to deplete the micromolecular content and to derive palmitoyl proteins which had similar solubility in water.

The standard glycinin which was taken through the dialysis steps showed no visible signs of precipitation. A glycinin control was taken through the entire scheme without 16:0-Osu and showed a 100% yield. Palmitoyl proteins A and B showed yields of 54 and 77%, respectively. Figure 1 shows the stages at which such precipitation occurred during the processing of palmitoyl protein A. The precipitation following urea removal (20%) was richly incorporated with 16:0 (data not shown). The exhaustive dialysis against water containing 10 mM 2-ME resulted in a further 26% precipitation of protein which was incorporated to 22 mol of 16:0/mol of glycinin. In both the cases, free 16:0 was negligible. In the case of palmitoyl protein B, the precipitation at both stages was apparently insignificant.

The yields of renatured glycinin from denatured and reductively denatured states have been reported to be 70

and 20%, respectively (Kitamura et al., 1977). Their results suggest that the formation of disulfide bridges between acidic and basic subunits is important in the formation of glycinin. Mori et al. (1979a) showed that renaturation of glycinin from the reductively denatured state was depressed significantly by the presence of other proteins (Mori et al., 1979b).

A number of workers have reported dissociation of a number of proteins as a result of succinylation (Klotz, 1967; Spande et al., 1970; Madisen and Cori, 1956). Hill and Kanarek (1964), however, saw no such dissociation in the case of fumarase which has 12 succinylable thiol groups. Palmitoyl protein B which was dissociated to the intermediary subunit stage in the presence of urea perhaps could not reassociate to form the 6 (acidic–basic subunit) structure of the glycinin molecule (Kitamura et al., 1976) following the removal of urea due to steric hindrance of the hydrophobic tails.

It is interesting to note that the palmitoyl proteins remain soluble in spite of their increased hydrophobicity. Palmitoyl protein B has been kept in 0.3% aqueous solution containing 0.025% NaN_3 at 4 °C for 60 days without any visible signs of precipitation. Aggregation of these palmitoyl proteins was perhaps prevented by the hydrophobic tails which were about 1 mol/mol of acidic subunit of palmitoyl protein A and intermediary subunit of palmitoyl protein B. For the purpose of calculation, the molecular weights of glycinin, intermediary subunit, and acidic subunit have been considered to be 360 000, 60 000, and 35 000, respectively.

Cumper and Alexander (1950) and Cumper (1953) explained that during foam formation, a monolayer of surface-denatured protein surrounded by liquid is rapidly adsorbed at the interface of the colloidal mixture, trapping air and forming bubbles. β-Casein, a structurally disordered, flexible protein, adsorbs rapidly at the air–water interface and gives good foamability (Graham and Phillips, 1976). Highly ordered globular proteins were difficult to surface denature and as a result had poor foamability. Horiuchi et al. (1979) showed that foam stability occurred when the hydrophobic region of proteins became situated at the interface, causing the molecule to resist migration to the aqueous phase.

Our foaming experiments (Table III) indicated that the slower sedimenting palmitoyl proteins (Figure 3) did not exhibit a higher surface-denaturing capacity in comparison with the standard glycinin which was a much faster sedimenting protein. This could indicate that the palmitoyl proteins were still highly ordered globulins and had not been denatured into a random conformation due to the incorporation.

The separate mechanisms that appear to be involved in formation of a stable emulsion include (1) reduction of interfacial tension, (2) formation of a rigid interfacial film, and (3) electrical charge (Lissant, 1974; Friberg, 1976). Stutz et al. (1973) have indicated that the first molecules of a surfactant introduced into a two-phase system act to form a monolayer; additional surfactant molecules tend to associate with each other, forming micelles, which stabilize the system by hydrophilic–lipophilic arrangements. Our emulsification experiments (Table III) indicated that both the palmitoyl proteins were surface active. The acidic (hydrophilic) subunit, i.e., palmitoyl protein A, attained a amphipathic nature following the equimolar incorporation of the hydrophobic ligand and seemed to be similar to palmitoyl protein B in spite of the likelihood that the intermediary subunit is naturally amphipathic due to its distinctly seperate hydrophilic and hydrophobic zone.

Watanabe et al. (1981) reported the enzymatically induced singular incorporation of leucine esters of different alkyl chain lengths to different food proteins. They, however, have reported no improvement in the emulsifying activity of succinylated soy protein isolate after incorporation.

Thin-layer chromatographic analysis of palmitoyl peptides gave a 16:0 to amino acid ratio of 1:5.2 in the case of palmitoyl protein A and 1:4.2 in the case of palmitoyl protein B. This indicated the nonhydrolyzability of the peptide bonds adjacent to the site of incorporation. The enzymatic hydrolysis employing the in vitro model system showed an apparently lower yield of almost all the amino acids. This indicates that the aminopeptidase (microsomal) was unable to effectively hydrolyze the above-mentioned bonds under the conditions of our in vitro model system.

It has been reported that the N^{ϵ}-acetyllysine is not released by pepsin and pancreatic proteases or that the N^{ϵ}-acetyl group of the lysyl residue is not deacetylated by pancreatin (Matoba and Doi, 1978). Matoba et al. (1980) observed that aminopeptidase (microsomal) had low activity against N^{ϵ}-acetyllysine and even lower activity against N^{ϵ}-succinyllysine. Recently, Groninger and Miller (1979) showed that acetyl- and succinyllysine residues of protein were absorbed into the intestines and that parts of both were detected in the urine of rats.

Finally, it is important to emphasize that although our observations indicate reduced digestibility of palmitoyl proteins, further kinetic study is required to establish this point. If the palmitoyl protein constitutes a small portion of the total intake, some lowering of digestibility could be of little consequence.

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Isolation and Identification of Additional Beef Flavor Precursors

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A beef fraction previously obtained by water extraction of lipid-free freeze-dried beef, ultrafiltration of the aqueous extract, and gel permeation chromatography of the ultrafiltrate was further fractionated by high-pressure liquid chromatography, and the components were identified by nuclear magnetic resonance and infrared spectroscopy. The compounds isolated and identified are glutamic acid, lactic acid, phosphoethanolamine, glycerol, creatine, and creatinine and represent 94% of the fraction. Some minor constituent amino acids were also identified.

The correlation of chemical composition with the flavor of beef remains elusive. Some flavor chemistry can be relatively simple, and perhaps the green bell pepper has become a classic example (Buttery et al., 1969), but most flavors are more complex. A significant complication in the case of beef are the modifications produced by cooking. Important nonvolatile flavor components are produced with heating (Tonsbeek et al., 1969), often with large flavor contribution (Tonsbeek et al., 1971). The array of volatiles with cooking is even more complex (Hirai et al., 1973; Mussinan et al., 1973; Wilson et al., 1973) and is not significantly simplified by different cooking conditions (MacLeod and Coppock, 1976, 1977) or by the study of model systems (Boeleus et al., 1974; Qvist and von Sydow, 1974). Since desirable meat flavor is developed by cooking, and most of the large number of aroma constituents thus produced probably play a significant role in the flavor, attempts to simplify the research lead to the study of precursors in the raw meat.

Lipids are important, both directly and as flavor precursors (Forss, 1969; Wasserman, 1972), but previous work at this laboratory was involved with water-soluble beef components. In addition to the amino acids, carbohydrates, and organic acids reported by Jarboe and Mabrouk (1974) in an aqueous beef extract, and their contributions to beef flavor directly and as precursors, as described, important precursor activity was presented for some gel permeation chromatography fractions of an aqueous beef diffusate (Mabrouk et al., 1969). The determination of the specific chemical composition of one of the more important of these fractions is the effort of this paper. Any beef fraction demonstrating precursor activity warrants analysis in order to ensure the successful formulation of synthetic mixtures, and this addition to the growing list of precursor compounds should assist toward this objective.

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EXPERIMENTAL SECTION

The gel permeation chromatography fraction having desirable aroma characteristics was prepared according to the methods described (Mabrouk et al., 1969; Mabrouk, 1973). Briefly, raw semimembranous beef muscle was blended with water, lyophilized, and extracted with petroleum ether. The lipid-free meat was then thoroughly extracted with water, and the aqueous extract was dialyzed [or ultrafiltered: Mabrouk (1973)]. The diffusate was then chromatographed on Sephadex G-25 fine and appropriate aroma characteristics were described. The second fraction thus obtained, averaging 67% of the diffusate, is the subject of this study.

High-Pressure Liquid Chromatography (HPLC). The beef flavor precursor (BFP) mixture obtained in this manner by Sephadex gel permeation chromatography was separated by using a Waters Associates ALC-100 chromatograph equipped with a Model 6000 solvent delivery