what organizational changes occurred at the molecular level that explain the different protein polymerization patterns observed in this study.

Abbreviations Used: MDA, malonaldehyde; TEM, transmission electron microscopy; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; G3PD, glyceraldehyde-3-phosphate dehydrogenase; AChE, acetylcholinesterase; HMWP, high molecular weight protein; DTT, dithiothreitol; DTNB, dithiobis(nitrobenzoic acid).

Registry No. AChE, 9000-81-1; G3PD, 9001-50-7; chloroform, 67-66-3.

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Lipophilization of α_{s1}-Casein. 3. Purification and Physicochemical Properties of Novel Amphipathic Fatty Acyl Peptides

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Fatty acyl peptides (A) were obtained from lipophilized α_{s1}-casein containing covalently attached caprylic (8:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids. The secondary structure was essentially the same regardless of the ligand size. Monomer weight was about 3500 and aggregation numbers were 3 (A_3), 6 (A_6), 12 (A_{12}), and 24 (A_{24}); the trimer was the most predominant. The driving force for initial aggregation (A_3) was spontaneous, but further aggregation seemed to be influenced by the ligand size; longer ligands led to larger aggregates. Surface tension (dyn/cm) decreased with the increase in ligand length, being lowest for 18:0-A. Surface tension equilibrium was attained within 60 min only when the ligand length was 18 carbons. The critical micelle concentration in 5 mM phosphate buffer, pH 6.8, was 1.5, 1.3, 1.2, 2.3, 1.8, and 1.7 M for 12:0-A, 14:0-A, 16:0-A, 18:0-A, 18:1-A, and 18:2-A, respectively. Foaming activity was higher when the ligand length was greater, and the foam density seemed to decrease with an increase in ligand length and in unsaturation.

Amphipathicity and micelle forming ability of proteins and peptides are properties that are being increasingly appreciated. The importance of hydrophobic interactions for the entry of certain peptides into cells has been noted (Uchida et al., 1980). Cytochrome b_5, a mitochondrial membranal enzyme, has been well investigated (Fleming et al., 1978) and appears to contain a hydrophobic taillike region distinctly separate from the hydrophilic milieu. A number of workers have reported the in vivo posttranslational covalent attachment of fatty acids to proteins (Henderson et al., 1983). Biologically active polypeptides have also been noted to contain covalently linked fatty acids (Carr et al., 1982).

It has previously been shown that fatty acids may be attached covalently to proteins by relatively mild methods (Haque and Kito, 1982, 1984), and the chemical, functional, and conformational effects of "lipophilization" (Haque et al., 1982; Haque and Kito, 19873a,b) have also been shown. However, a clearer understanding of the effect of lipophilization would ensue if the subject of study were peptides. On the other hand, even though previous work (Haque and Kito, 1983b) has shown Lys-32 and/or -34 to be the most reactive nucleophile in α_{s1}-casein, confirmation was desirable. And finally, the bulk purification of highly amphiphilic peptides containing fatty acyl anchors has not been attempted. Most efforts have been made using preparative HPLC (Tanabe et al., 1979), and conventional methods of peptide purification, such as ion exchange or

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gel chromatography, have failed to give good results due to excessive hydrophobic interaction of the hydrophobic anchors. This chapter deals with some properties of a novel amphiphilic peptide that was obtained from α_{s1}-casein in high purity, as determined by high-performance liquid chromatography, following enzymatic hydrolysis of fatty acyl α_{si}-casein.

EXPERIMENTAL SECTION

Materials. Carboxypeptidase A (EC 3.4.17.1), linoleic acid (GC grade), dansyl chloride, dansyl amino acid standards, β-2-thionyl-DL-alanine, and γ-aminobutyric acid were from Sigma Chemical Co., St. Louis, MO. The ultrafiltration membrane (PM-10) was from Amicon, Lexington, MA, and filters type Ekicrodisc 13 and 13CR of pore size 0.45 m were from Gelman Science, Japan. *p*-Toluenesulfonic acid, Pronase E (1:1000 000), pancreatin NF, trifluoroacetic acid (TFA) (special grade), phenyl isothiocyanate (special grade), propionic acid, redistilled hydrochloric acid (HCl), heptadecanoic acid, acetonitrile (GC grade), tetramethylammonium bromide, ethylene glycol, and pinacyanol chloride were from Nakarai Chemicals, Kyoto. Sodium 1-butanesulfonic acid and 3-(2-aminoethyl)indole were from Tokyo Kasei, Tokyo. Molecular weight markers of molecular weight 2500–17 000 (myoglobins) were from Fluka AG Chemische Fabrik, Switzerland, and the rest from Pharmacia Chemicals, Sweden. α_{s1}-Casein was prepared as mentioned before (Haque and Kito, 1983a). Glass-distilled, deionized water was purchased from Wako Chemicals, Osaka. Polyamide layer sheets (15 × 15 cm) were from Seikagaku Kogyo Co., Ltd., Tokyo. All other reagents and solvents were of analytical grade.

Methods. *Preparation of Active Esters.* *n*-Hydroxysuccinimide esters (fatty acyl-Osu) of *n*-caprylic (8:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids were prepared as described previously (Haque and Kito, 1982). 18:1-Osu, which was oily at room temperature, gave a waxlike material after being solubilized in diethyl ether and dried in a stream of nitrogen. In the case of 18:0-Osu, following the reaction, filtration, and evaporation of the reaction medium, as the product was insoluble in ethyl acetate, the usual solvent, chloroform–methanol (2:1 v/v) was used instead. 18:0-Osu was recrystallized from ethyl acetate. All active esters were thoroughly desiccated over phosphorus pentoxide and stored at −20 °C.

Bulk Preparation of Lipophilized α_{s1}-Casein. The lipophilization of pure α_{s1}-casein was as described before (Haque and Kito, 1983a) except that 0.15 M NH_4OH was used instead of borate buffer. The content of ethanol in the reaction mixture was also decreased slightly to 65% (130 mL), and the protein content was increased to 1% (2 g, 4.6 μmol). The lysine to active ester ratio was set at 1.5, and so the content of active ester was 1.9 mmol (Haque and Kito, 1982).

Enzymatic Hydrolysis. The reaction mixtures from the above preparations were dried at 30 °C in vacuo in a rotatory evaporator and then solubilized in 0.1 M ammonium bicarbonate. The pH was adjusted to 7.8 with acetic acid, and then pancreatin NF of a weight ratio of 1:20 (with protein) was added and the solution incubated at 37 °C for 12 h. The solution was then passed through a PM-10 membrane filter and the permeate incubated again with the same weight ratio of Pronase E for 4 h at 40 °C, pH 7.5. Both enzymes were solubilized in the same buffer (1% solution) at 4 °C for 1 h and then centrifuged prior to use. Following the second incubation the solution was again passed through the membrane filter and then freeze-dried to give a free flowing powder that was termed "crude peptide preparation".

Purification of the Novel Amphiphilic Peptide. The crude peptide preparation was mixed for 1 min with a Polytron (type PT10/35), stirred overnight in 200 mL of methanol (99.6%) at 4 °C, and then kept in a sealed container at 20 °C for 6 h following which the solution was centrifuged (13000 *g*, 10 °C, 20 min). A pale, slightly yellowish solution was obtained and again kept at −20 °C for 12 h. Centrifugation yielded a trace amount of precipitate that was discarded, and the solution was equilibrated to 20 °C in a desiccator over silica gel. A half volume of diethyl ether, that had previously been kept over KOH pellets (48 h) to reduce the peroxide content was added gradually while stirring gently to give a thick white precipitate that was separated by centrifugation (2000*g*, 25 °C, 5 min) to give a fraction hereafter to be termed "A-rich fraction". The ether content of the supernatant was further increased to a ratio of 1:1 with methanol, and a second precipitation, hereafter termed "C-rich fraction", was collected by centrifugation and stored at −20 °C for future use. The A-rich fraction was then solubilized again in 100 mL of methanol and reprecipitated with an equal volume of ether followed by centrifugation (5000*g*, 25 °C, 10 min). The precipitate was immediately solubilized in 10 mL of freshly distilled water (neutral pH), and 190 mL of methanol was added to give a clear solution that was equilibrated in an ice bath for 1 h, precipitated with 1.5 volumes of ether, again held at 0 °C for 1 h, and then centrifuged (8000*g*, 0 °C, 10 min). The 95–97% pure peptide (fraction A by HPLC as stated below) thus derived was then solubilized in 50 mL of distilled water, the pH adjusted to 4 with 0.1 N HCl, and the solution extracted once with water-saturated 1-butanol, 4 times with water-saturated ethyl acetate, and finally with petroleum ether. The volume of organic solvent used in each of the above cases was 10, 25, and 50 mL, respectively. The organic extract was discarded and TFA was then added to the aqueous layer to a 1% level (v/v) and the solution was again extracted exhaustively (6 times) with water-saturated 1-butanol. The aqueous layer was discarded. The pH of the 1-butanol extract was adjusted to around neutral with NH_4OH, and then the extract was dried in vacuo in a rotatory evaporator at 30 °C and desiccated in vacuo over phosphorus pentoxide for 24 h. The fatty acyl peptides thus derived have been termed 8:0-A, 12:0-A, 14:0-A, 16:0-A, 18:0-A, 18:1-A, and 18:2-A according to the fatty acyl ligand attached to it. In the case of the unsaturated fatty acids (18:1 and 18:2), all solvents and reaction mixtures from the very beginning were saturated with nitrogen and the top of reaction vessels and storage containers were flushed with nitrogen to minimize contact with oxygen.

High-Performance Liquid Chromatography (HPLC). An Hitachi 655-15 twin piston pump attached through an appropriate column to an Hitachi variable-wavelength UV monitor, Type 638-41 (set at 220 nm), and an Hitachi Chromato-Processor, Type 833, was used for all experiments. All samples were passed through a Ekicrodisc (13) filter prior to injection, and all solvents were degassed by sonication.

(*a*) *Molecular Weight Determination.* A TSK-GEL G3000SW (Toyo Soda, Tokyo) controlled pore hydrophilic glass bead column was used (60 cm × 7.5 mm i.d.) for the molecular weight experiments. The flow rate was 0.5 mL/min at 25 °C. The eluting buffer for detecting the molecular weight of the monomeric form of the peptides, and for the molecular weight markers, was 33.3 mM sodium phosphate, pH 6.8, containing 0.4 M NaCl and 5%

(v/v) ethylene glycol, the total ionic strength (I) being 0.5. In the case of the determination of the aggregate number, the buffer was 5 mM sodium phosphate (I = 0.015), pH 6.8. The flow rate was 0.5 mL/min at 25 °C and detection was by absorption at 220 nm and the injection volume was 5 L of a 10% (w/v) solution of the fatty acyl peptide A.

(*b*) *Determination of the Purity of Peptides.* An Ultrasphere ODS column (4.6 i.d. × 250 mm) (Altex Scientific, Inc., Berkley, CA) was used for determining the purity of the peptides. The solvent used was 30% (v/v) acetonitrile in 0.1 M sodium phosphate, pH 5.8, containing 5 mM sodium 1-butanesulfonate and 5 mM tetramethylammonium bromide. The flow rate was 1 mL/min at 25 °C.

N- and C-Terminal Sequencing. N-Terminal sequencing was by Edman degradation, dansylation, development on polyamide sheets, and subsequent visualization under UV (Gray, 1972). C-Terminal sequencing was carried out by using carboxypeptidase A (Amber, 1972).

Amino Acid Analysis. An Hitachi 835 high-speed amino acid analyzer using the citrate buffer system of Spackman et al. (1958) was used for the quantitative estimation of amino acids in the protein–peptide hydrolysates. Hydrolysis was done with 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole at 110 °C for 24 and 48 h. Internal standards were β-2-thionyl-DL-alanine and γ-aminobutyric acid.

Detection of Fatty Acids. Aliquots of the peptide samples (20 mg) were hydrolyzed with 12 N HCl–propionic acid (1:1 v/v) for 30 min at 130 °C. Margaric acid (17:0) was used as the internal standard. The hydrolysate was dried in vacuo at 60 °C, resolubilized in 1 mL of 1 N HCl, and then extracted 3 times with the same volume of diethyl ether. The ether was evaporated in a stream of nitrogen, and methylation was carried out with freshly prepared diazomethane in ether containing 10% methanol. Detection was by GLC as mentioned earlier (Haque et al., 1982).

Determination of Surface Tension. Surface tension was determined by using a Wilhelmy plate type surface tensiometer (Kyowa Model CMVP type A3) fitted to a Yokogawa printer, Type 2543. Readings were printed out every 2 s for the first 2 min and then at 10-min intervals. Surface tension at 10 s and at 10, 20, 30, 40, 50, and 60 min were fed to a computer (Facom M 182AD) for negative logarithmic regression of the data. The sample size was 5 mL at 25 °C. Glass Petri dishes were equilibrated to the experimental temperature for 15 min following which the temperature-equilibrated, freshly prepared peptide solution (0.1% w/v) was poured into the dish and the experiment was started immediately. The solvent was 5 mM sodium phosphate, pH 6.8 Equilibrium surface tension has been defined as the surface tension (dyn/cm) that is attained and maintained for at least 10 min within 1 h after immersing the Wilhelmy plate.

Whipping Property. The foam activity (FA), density (FD), and stability (FS) were determined as stated previously (Haque and Kito, 1983a). The concentration of the solution was 0.1% (w/v) in 5 mM sodium phosphate, pH 6.8.

Circular Dichroism. The secondary structural content was determined as stated before (Haque and Kito, 1983b).

Micelle Formation. (*a*) *Micelle in Water.* Dye adsorption experiments were performed to determine the critical micelle concentration (cmc) of the fatty acyl peptide As. All solvents used were deaerated in vacuo and bubbled with nitrogen prior to use. Pinacyanol chloride (5 mg) was dissolved in 200 mL of 5 mM sodium phosphate buffer, pH 6.8, and the solution was passed through a filter paper (Whatman No. 1) and kept in a flask wrapped in black paper. All the experiments were conducted with the same solution and within as short a period as possible in a dimly lit room to prevent photodegradation of the dye (Timbers and Lingaflter, 1949). Experiments were carried out at a constant concentration of the dye by the gradual dilution method as follows: An initial solution of the fatty acyl peptide fraction was made directly with the dye solution. Following the spectrophotometric measurement, a definite amount of the solution was removed, and the same amount of the dye solution was added and mixed with a vortex mixer to give the next sample. The initial sample was used as a standard to correct for instrumental drift with time. Measurement was at 610 nm against the buffer, at 20 °C.

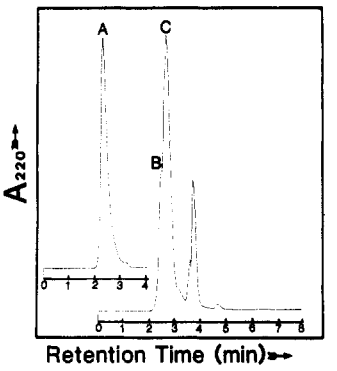

Figure 1. HPLC of fatty acyl peptides. "A" shows the HPLC (see Methods) profile of fatty acyl peptide A. "B" and "C" are profiles of fractions that remained after separation of (A).

(*b*) *Reverse Micelle.* The ability of the fatty acyl peptide As to form a "pool water system" within a reverse micelle was investigated by using TCNQ (Kanamoto et al., 1981). A total of 50 mg of TCNQ was vigorously agitated (Polytron, type PT10/35) at 40 °C for 2 min followed by filtration to remove the insoluble residue. Three milliliters of filtrate was used for each of the following tests. The fatty acyl peptide A solution was mixed with deionized water to give a 10% (w/v) solution, 1 μL (100 μg) was added to the TCNQ solution and sonicated for 1 min (Branson cell disrupter 200; 20 kHz) at 20 °C, and then the absorbance at 480 nm was recorded immediately.

RESULTS

Purity of Peptide. Figure 1 shows the HPLC profile, using a ODS column (see Methods), of the purified peptide A fraction and the other fractions (B, C, etc.) that remained in the supernatant after "A" separation. Fraction A was obtained in all cases (8:0-A, 12:0-A, 14:0-A, 16:0-A, 18:0-A, 18:1-A, and 18:2-A) in >99%g purity as judged from the HPLC processor data. The HPLC profile was almost the same for all fatty acyl peptide A fractions. The retention time of A was 2.3 min as compared to 2.6 and 2.8 min for B and C, the other major fractions. All HPLC experiments were repeated at least 3 times to verify repeatability.

Molecular Weight. Figure 2 shows the monomer weights and aggregation number of the different fatty acyl peptide As. The molecular weights of all the fatty acyl peptide As (A_1) were seen to be the same at about 3500. The gel filtration results gave a highly repeatable and similar aggregation number for all fatty acyl peptide As studied except for 8:0-A, which showed aggregation that was not as orderly and well-defined as in all the cases and has therefore been excluded from this part of the study. The molecular weights of the aggregates were 10 500 (A_3),

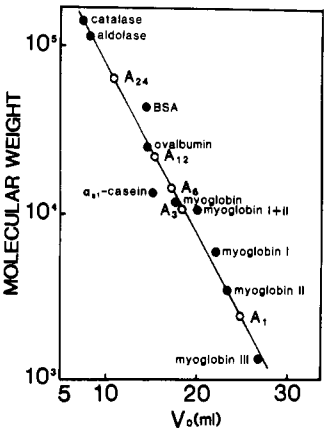

Figure 2. Molecular weight of fatty acyl peptide A and its aggregates. A_1, monomer of fatty acyl peptide A; A_3, A_6, A_{12}, and A_{24}, aggregation number of 3, 6, 12, and 24.

Table I. Percentile Distribution of Aggregates[a]

fatty acyl peptide A	A_1	A_3	A_6	A_{12}	A_{24}
8:0-A					
12:0-A	8	80	9	1	
14:0-A	7	65	18	5	2
16:0-A	6	58	20	6	5
18:0-A	5	59	18	5	9
18:1-A	5	62	17	5	8
18:2-A	6	64	16	5	7

[a] At 25 °C in 5 mM sodium phosphate, pH 6.8.

Table II. Amino Acid Composition[a]

amino acids, mol/mol	amino acids in peptides	amino acids in segment 26–52[b]
Asx	3.0	3.0
Thr	1.0	1.0
Ser	2.0	3.0
Glx	6.0	6.0
Pro	2.0	2.0
Gly	1.5	2.0
Ala	1.0	1.0
Val	1.5	2.0
Ile	0.8	1.0
Leu	0.8	1.0
Phe	1.1	2.0
Lys	3.0	3.0

[a] Mean value (n = 3) of p-toluenesulfonic acid hydrolysis. [b] Segment from α_{s1}-casein (Mercier et al., 1971).

24 000 (A_6), 41 500 (A_{12}), and 83 000 (A_{24}). A trace amount showed molecular weight of 65 000 (A_{18}) (data not shown). The relative percentage of the different aggregates is varied with the ligand size. This is shown in Table I.

Amino Acid Composition. Table II shows the amino acid composition of the fatty acyl peptide As. All of the peptides had the same amino acid composition, and the average number of incorporated fatty acid ligand was one in all cases of 12:0-A, 14:0-A, 16:0-A, and 18:0-A analyzed. 8:0-A could not be measured due to low extractability from the aqueous system. 18:1 and especially 18:2 were readily decomposed under the hydrolytic conditions used. The N-terminal residues were Ala-Pro-Phe-. The C-terminal residues were Gln-Asp-Glu-. Judging from the primary sequence of α_{s1}-casein (Mercier et al., 1971), we conclude that the peptides were segment 26–52, consisting of 27 amino acid residues. The calculated molecular weight of these peptides (including one fatty acyl residue) compares very well with the monomer weight determined by gel filtration (Figure 2).

Table III. Secondary Structural Content[a]

peptides	α helix	β[b] anti	β[b] para	turn[c] I	turn II	turn III	turn T	others	RI[d]
8:0-A	3.1	34.3	1.6	9.5	5.4	2.5	5.9	37.7	0.17
12:0-A	3.6	34.2	1.5	9.5	5.3	2.5	5.8	37.6	0.16
14:0-A	6.5	31.5		9.3	6.8	2.5	5.9	43.3	0.10
16:0-A	6.0	29.5		8.9	5.6	2.4	5.8	42.0	0.11
18:0-A	7.9	29.5		8.8	5.6	2.5	5.7	40.6	0.08
18:1-A	6.2	30.7		9.2	5.9	2.6	5.8	41.1	0.10
18:2-A	6.9	33.6		9.6	6.6	2.8	5.9	39.5	0.10

[a] At pH 6.8, 20 °C, in 5 mM sodium phosphate. [b] β anti = β antiparallel pleated structure; β para = β parallel pleated structure. [c] β turns as per criteria of Chou and Fasman (1978). [d] RI = reliability index = [CD(calculated) − CD(observed)]/CD(observed).

Table IV. Whipping Property and Surface Tension

peptides	FA	FS	FD	σ[a]	σ_e[b]	t_e, min[c]
8:0-A				54.2 ± 0.4[d]		
12:0-A				45.9 ± 0.8[e]		
14:0-A				44.2 ± 0.9[e]		
16:0-A	107	92	6.25	38.9 ± 1.0[g,i,j]		
18:0-A	135	99	3.40	37.4 ± 0.0[f–h]	37.5	0.25
18:1-A	110	98	2.20	38.7 ± 1.9[h,j,k]	39.4	32.00
18:2-A	123	98	1.80	38.3 ± 0.5[f,i,k]	37.9	47.00

[a] Mean ± SD values (n = 7) (see Methods) of surface tension. [b] Equilibrium surface tension. [c] Time at which equilibrium surface tension is attained. [d] Different from all at $P < 0.001$. [e] Different from each other at $P < 0.01$ and from the rest at $P < 0.001$. [f] Different at $P < 0.01$. [g] Different at $P < 0.02$. [h] Different at $P < 0.20$. [i] Different at $P < 0.30$. [j,k] Similar at $P < 0.50$.

Secondary Structure. Table III shows the structure of the fatty acyl peptide As. The secondary structure (Chou and Fasman, 1978) of the different fatty acyl peptide As seems to vary little.

Micelle Formation. The cmc of the different fatty acyl peptide As determined in 5 mM sodium phosphate, pH 6.8, at 20 °C were 1.5, 1.3, 1.2, 2.3, 1.8, and 1.7 M for 12:0-A, 14:0-A, 16:0-A, 18:0-A, 18:1-A, and 18:2-A, respectively. 8:0-A was excluded from this experiment since the change in absorbance was not so clear. It may be noted that the cmc of all the fatty acyl peptide As was very low and seemed to increase slightly with the increase in ligand size (18:0-A). Unsaturation (18:1-A and 18:2-A) seemed to reverse the trend. The TCNQ experiments gave A_{480} results that were 0.02, 0.02, 0.03, 0.095, 0.15, 0.02, 0.21, and 0.16 for the blank (no peptide), 8:0-A, 12:0-A, 14:0-A, 16:0-A, 18:0-A, 18:1-A, and 18:2-A, respectively. The efficiency of reverse micelle formation under our conditions (see Methods) was therefore seen to be in the following order: 18:1-A > 18:2-A > 16:0-A > 14:0-A. 8:0-A, 12:0-A, and 18:0-A were not effective.

Surface Tension. Table IV shows the surface tension of the different fatty acyl peptide As. The statistical significance is shown in the footnote. 12:0-A and 14:0-A showed a similar surface tension reducing tendency but a larger ligand size increased the surface activity significantly. 18:0-A was only peptide that attained equilibrium surface tension within 1 min of dipping the Whilhelmy plate. Unsaturation seemed to decrease surface activity slightly.

Whipping Property. The FA (Table IV) showed a clear relationship with the ligand size. The larger the ligand size, the higher the FA. However, there was a sharp drop in the FA when one double bond was added (18:1-A).

DISCUSSION

The fatty acyl peptide As were all obtained in high purity as is apparent from the sharp single peak that was

obtained by HPLC using a ODS column (Figure 1). Free amino acids, especially Lys and Arg, were removed by the repeated extraction with water-saturated ethyl acetate (see Methods). The 1-butanol extraction at pH 4 removed small peptide contaminants. Initial experiments showed that the fatty acyl peptide As did not move into the 1-butanol phase under this condition regardless of the ligand. TFA (0.1% v/v), on the other hand, was seen by HPLC to facilitate extraction of fatty acyl A peptides by water-saturated 1-butanol. This enhanced lipophilicity in the presence of TFA is the result of ion-pair partition and not simply due to the acidic condition since no such partitioning was seen to occur even when 0.1% HCl, acetic acid, or formic acid was used (data not shown). The extraction steps were done as quickly as possible to reduce the time that the peptides would be exposed to acidic conditions. The purified peptides were highly hygroscopic, and care had to be taken to always keep them dry. The anionic ion pair reagent, sodium 1-butanesulfonic acid, and the cationic ion pair reagent, tetramethylammonium bromide, greatly improved the sharpness and reproducibility of the HPLC study. It is assumed that the RSO_3^- anions block the residual OH groups of the RP packing and also possibly form ion pairs.

The gel filtration study (Figure 2) shows that the peptides tend to exist in a highly aggregated form and the aggregation does not appear to be random or disorderly. Repeated experiments showed the same aggregation numbers as would be expected in the case of well-defined hydrophilic–lipophilic arrangements. It is interesting to note that the first unit in the series of aggregation is a trimer and not a dimer or tetramer. This oligomer was the most predominant species (Table I). Aggregation of the fatty acyl peptide As was seen to proceed until A_{24} when the counter ion concentration was low ($I = 0.015$). When the ionic strength (I) was high ($I = 0.30$), the monomer was predominant and A_3 was about 8%. It may be noted that when the ionic strength was even higher ($I = 0.5$), aggregation was not detected (data not shown). From Table I it appears that the fatty acyl peptide As tend to exist more in the aggregated A_3 form than in the monomeric A_1 form and this initial association seems to be primarily ionic in nature. However, as is apparent from the percentile distribution of the aggregates (A_6, A_{12}, and A_{24}), the length of the hydrophobic ligands dictates the size of the aggregate. The longer the ligand, the greater the occurrence of the larger aggregates. It appears that the ligand should be 14:0 or longer for the A_6, A_{12}, and A_{24} to form. Even though 8:0-A had the same the amino acid composition, the aggregation did not appear to have coordinated hydrophilic–lipophilic arrangement. Hence, it is evident that the length of the hydrophobic ligand is important for the formation of micellar aggregates. Hydrophobic patches alone are not enough to control aggregation. The spacial flexibility and the geometry may be crucial for micelle formation. It may be noted that sodium dodecyl sulfonate (0.2% w/v) is not suitable for the gel filtration of the fatty acyl peptide As or the clear separation of the peptide markers. Perhaps it participated in mixed micelles. Triton X-100 also complicated results. While working with sphingomyelin, Yedgar et al. (1974) concluded that Triton formed mixed micelles and acted by overcoming the low surface curvature of the bilayer so that particles can be formed with sufficient curvature to close upon each other in a small radius. Above the critical concentration in water, bile salts form aggregates that are of two types: primary and secondary. The former may be 2–10 in number whereas the latter is 12–100 (Helenius and Simons, 1975).

The amino acid analyses of the fatty acyl peptide As show that the peptides were composed of 27 amino acid residues and contained the most reactive site in α_{s1}-casein (Lys-32 and/or -34) (Haque and Kito, 1983a). The slightly low total analyzed value is because there was a small loss of hydrophobic amino acids from the hydrolysates. It is obvious that one of the bitter peptides isolated from casein by Matoba et al. (1970) was segment 23–34 of α_{s1}-casein (Mercier et al., 1971). It is highly probable that the covalent attachment of the fatty acyl ligands to Lys-32 or -34 makes the enzymatic cleavage of peptide bond 34–35 difficult, deriving the longer segment. The fatty acyl peptide As were not bitter.

The cmc of the fatty acyl peptide As seem to be very low as described in the text. Palmitoyl-CoA, which is about $^1/_3$ the size of the fatty acyl peptide As, has a cmc that is about 2 times higher (3–4 μM) (Zahler et al., 1968). A clearly observed drop in the absorbance was not seen for 8:0-A. Even though very slight, there is to be a tendency for the cmc to increase as the ligand size increases to 18.0. Contrary to the observations in water, the behavior in heptane is different. Here the efficiency is highest for 18:1-A followed by 18:2-A, 16:0-A, and 14:0-A. 18:0-A did not form a pool water system as was the case with 8:0-A and 12:0-A. This may be due to the difference in the dielectric constant of the two bulk systems. The dielectric constant of the bulk water at 25 °C is about 80 as compared to only 1.92 for heptane.

The surface tension (Table IV) decreases as the ligand size increases. This happens in spite of the fact that the monomeric form (A_1) decreases as the ligand length increases (Table I). The surfactant monomer is essential for the formation of the surface monolayer that in turn reduces surface tension. Even though fewer, peptides with longer fatty acyl moieties are more surface active. Table IV also shows that only 18:0-A, 18:1-A, and 18:2-A attained equilibrium surface tension. 18:0-A was unusually stable and attained equilibrium surface tension within only 1 min. The efficiency factor of surface tension reduction increases with an increase in the hydrophobic character of the surfactant (Rosen, 1974). On the other hand, we are aware that the larger the surface concentration attained by the surfactant, the larger the cmc and the smaller the bulk concentration necessary to depress the interfacial tension (Rosen, 1978).

The foaming experiments have indicated (Table IV) that longer ligands are more effective and that unsaturation decreases the FA but FS remains the same. As mentioned above, the equilibrium surface tension was observed only in those fatty acyl peptide As that showed high FA and FS (18:0-A, 18:1-A, and 18:2-A). The surface tension of these peptides was also significantly lower. The FD, however, seems to decrease with the ligand size and the degree of unsaturation. Further detailed study is required to establish this point.

This part of the study indicates that the attachment of fatty acyl moieties to peptide may greatly improve surface activity and has a bearing on the cmc and aggregation number. The whipping property is affected by this covalent incorporation.

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Interaction of Phosphatidylcholine Vesicles with Soybean 7S and 11S Globulin Proteins

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Soy phosphatidylcholine (PC) vesicles prepared by a reverse-phase evaporation method in the presence of ^{14}C-labeled sucrose were mixed with 7S or 11S globulin solutions of varying concentrations. Since both protein preparations contained iron, controls in 2.5–5 mM $FeCl_3$ and controls without protein or iron saturated with air or nitrogen were used to estimated oxidative degradation of lipid species. Both 7S and 11S reduced the level of sucrose entrapment, but results varied widely among preparations. The level of reduction had no significant dependence up globulin type or protein concentration. Ferric ion reduced the level of sucrose encapsulation by approximately 25% after 4–5 days at 4 °C. With *N*-[^{14}C]-methyl-labeled soy PC, liposomes preformed by sonication were added to protein solution at known mole ratios of PC to protein. After about 12-h storage at 4 °C, preparations were subjected to an ultracentrifugal separation procedure. Gradient fractions were compared to those of a liposome control for label content. These results confirm the existence of protein–lipid interactions, with the inference that the extent of association is different for both globulin preparations.

The 7S and 11S soy protein globulins, so designated on the basis of sedimentation behavior, comprise about 70% of the total seed protein content, with a major protion of the proteins located in protein bodies within a matrix of lipid spherosomes. Although lacking specific biological activity, each globulin is composed of smaller subunits, and the 7S globulins are glycoproteins containing mannose and *N*-acetylglucosamine residues. The physical and chemical properties and amino acid composition for these globulins were reviewed by Wolf (1972), Hill and Breidenbach (1974), and Koshiyama (1983).

Soy globulin–phosphatidylcholine (PC) complexes are formed when systems containing protein and PC are sonicated together (Kanamoto et al., 1977). Reports of similar associations of liposomes with either 7S or 11S proteins (Ohtsuru et al., 1979; Ohtsuru and Kito, 1983) implied that the lipid interactions occur at hydrophobic regions within the protein and lead to alteration in protein conformation and that the association does not take place at specific amino acid residues.

The general effects of protein interaction with lipid vesicles have been studied (Guo et al., 1980: Ohki and Leonards, 1982; Schenkman et al., 1981; and many citations given therein). Large Liposomes have been a means of delivering nucleic acid polymers to plant protoplasts (Lurquin et al., 1981; Matthews and Cress, 1981; Lurquin and Sheehy, 1982) and have served as an immobilizing substrate for trypsin (Goldmacher, 1983). By using the encapsulating property of lipid vesicles, this investigation reevaluated the extent of soy globulin–soy PC interactions and considered the possible detrimental effects produced by protein–lipid interaction and oxidation of lipid residues upon the soy phospholipid vesicle dynamics.

MATERIALS AND METHODS

Soybean Materials. Soy PC type 111-S in chloroform solutions (100 mg/mL) was purchased from Sigma Chemical Co., St Louis, MO. *N*-[^{14}C]-Methyllabeled PC, prepared by the procedure of Patel et al. (1979) from soy phosphatidylethanolamine, was supplied by C. G. Crawford of this laboratory as a chloroform solution (ca. 12 mg of PC/mL). The 7S and 11S protein fractions, isolated by the method of Thanh and Shibasaki (1976) and Thanh et al. (1975), were provided by D. H. Honig of this laboratory.

Reagents and Solvents. Ficoll, a nonionic polymer of sucrose, types 70 and 400 DL were obtained from Sigma Chemical Co., St Louis, MO. Aqueous solutions of [^{14}C]sucrose (250 μCi) were purchased from the Amersham Corp., Arlington Height, IL. Maxifluor, a liquid scintillation cocktail for use with aqueous media, was from the J. T. Baker Chemical Co., Phillipsburg, NJ. All other chemicals used were commercially available and of reagent-grade quality. Organic solvents were used directly or redistilled.

High-Pressure Liquid Chromatography (HPLC) of Lipids. A C-18 μBondapak stainless steel semipreparative

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