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Chemical and Physical Lipophilization of Proteins

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With a view to enhance the amphipathic nature of food proteins, chemical and physical modification were carried out. Soy glycinin and α_{S1}-casein were lipophilized by chemically attaching naturally occurring fatty acids to them. The covalent attachment of fatty acyl residues to these proteins caused an increase in their emulsification activity. Soy proteins and the maize protein, zein, were associated with soy lecithin and phosphatidate, respectively, by sonication. The emulsification activity of phospholipid-protein complexes was greatly increased after they were treated with 50% ethanol or enzyme digestion.

Proteins in soybeans and maize have become widely used for feeding not only to poultry and cattle but also to swine. When we recognize a worldwide protein shortage, emphasis is centered on the development of new technologies to make plant proteins available and acceptable as human food. However, poor functional properties of these proteins limit their widespread use. Hence, we have to endow plant proteins with improved properties in order to increase their utilization as food materials.

The existence of hydrophobic amino acid side chains on protein molecules suggests the possibility of interaction between protein and small molecules containing hydrocarbons, such as hydrocarbons themselves, simple amphiphiles, and biological lipids. Interactions of this type presumably play an important role in the formation of functioning biological membranes. In the food systems too, lipid-protein interactions have long been recognized as playing a critical role in determining the functionality of food proteins. The hydrophilic shell of the protein has hydrophobic patches even in the native state. However, not only does the covalent attachment of a long-chain fatty acid create a new hydrophobic patch; it also introduces a tail-like ligand unless it is completely withdrawn within the protein core. In addition, complexes which are formed by association of phospholipids with proteins create a similar arrangement. The term"lipophilization" has been used to denote a general increase in the surface hydrophobicity of the protein molecule brought about by attachment of hydrophobic ligands in an effort to increase the affinity of proteins for relatively apolar molecules or amphiphiles.

Here I summarize recent papers published from this laboratory on chemical and physical attachment of fatty acyl residues and phospholipids to food proteins.

COVALENT ATTACHMENT OF FATTY ACYL RESIDUES TO GLYCININ (1,2)

N-Hydroxysuccinimide esters of lauric, myristic, palmitic and oleic acids were used as lipophilic electrophiles and were synthesized by reacting with equimolar amounts of dicyclohexylcarbodiimide in tetrahydrofuran (THF) (1:10, w/v) for 2 hr and overnight at 0 C and 20 C, respectively. The resulting dicyclohexyurea was removed by vacuum filtration, and the filtrate was evaporated under reduced pressure to be redissolved in the same volume of ethyl acetate. The products thus synthesized were recrystallized.

The lipophilization of glycinin was carried out by gradually adding the active ester (1:3, w/w, ester to protein) dissolved in THF (10% of final reaction medium) to a 0.5% protein solution in 33 mM potassium phosphate buffer (KPB) containing 0.4 M NaCl (μ=0.5) and 8 M deionized urea. Protein which had been stored in standard buffer (3) was freed of 2-mercaptoethanol by column chromatography using Sephadex G-25 just prior to use. The mixture was agitated for 30 min using a "swinging arm agitator" at 25 C following the elevation of the pH to 9.0. The pH dropped to about 8.5 at the end of the incubation period and the solution was dialyzed at 4 C overnight against 33 mM KPB containing 1 M NaCl, pH 7.6, following which it was washed five times with chilled (4 C) diethyl ether and dialyzed exhaustively for three days against water. The lauroyl, myristoyl, palmitoyl and oleoyl glycinins thus derived were freeze dried, and the degree of fatty acid incorporation was determined by gas liquid chromatography using a glass column packed with Silar 10 C on Chromosorb W.

TABLE 1.

Functional Properties of Palmitoyl Glycinin[a]

Sample	Foam activity	Foam stability[b]	relative emulsifying activity[c]
Glycinin	101	60	100
Palmitoyl glycinin	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.

Samples were acid hydrolyzed on 6 N HCl at 110 C for 4 hr and extracted with diethyl ether followed by methylation with diazomethane prior to GLC analysis. Covalent attachmnt was confirmed by checking the TLC mobility of Pronase-E hydrolyzed lipophilized peptides as follows: The sample (1% protein solution at pH 7.0 in 33 mM KPB containing 0.4 M NaCl) was hydrolyzed for 3 hr at 37 C with Pronase-E (50 units/mg protein) and the pH was then lowered to 6 to facilitate extraction with the solvent chloroform-methanol-n-butanol (2:1:1, v/v/v). The extract was then concentrated in vacuum, applied to a TLC plate and developed along with parellel references with chloroform-methanol-acetic acid (80:20:1, v/v/v). Fatty acids and active esters, if any, traveled to the solvent front. Appropriate areas, as determined by visualizing parallel references with ninhydrin, were scraped off to be acid hydrolyzed followed by the same method for the determination of fatty acid methyl esters.

The experiments showed that lipophilization of soybean glycinin is possible via base catalyzed ester exchange using N-hydroxysuccinimide esters of fatty acids as the electrophilic lipophiles.

Foam activity and stability. Foam activity and stability are shown in Table 1. Palmitoyl protein (1 mol palmitoyl residue/glycinin) showed higher foam stability and a slightly higher foam activity than unmodified glycinin.

Emulsifying activity. The comparative emulsifying activity, as seen in Table 1, shows that the palmitoyl protein has significantly higher emulsifying activity than glycinin. The results shown are for 0.4% protein concentration. The oil to water ratio was 1:5. At higher protein concentrations, it was observed that the amount of oil present was the limiting factor for the palmitoyl protein, contrary to the standard glycinin in which case the protein concentration was more important. The palmitoyl protein showed emulsifying activity which was 266% that of standard glycinin.

COVALENT ATTACHMENT OF FATTY ACYL RESIDUES TO α_{s1}-CASEIN (4-6)

The concentration of α_{s1}-casein in the reaction mixture was fixed at 0.2 mM, and the reaction temperature was 30 C. The solvent used was 50 mM sodium borate (adjusted to pH 9.0 with HCl) and ethanol (99.5%) in the ratio of 2:8. In the case of bulk preparation, the degree of incorporation was limited by controlling the mole ratio of N-hydroxysuccinimide ester of palmitic acid (16:0-Osu) to lysine residues in α_{s1}-casein. The molar content of lysine in the protein was considered to be 14 (7). Finely powdered 16:0-Osu was quickly dissolved by vigorous stirring in the ethanol component of the solvent system, which had been preheated to 35 C and then immediately mixed with the protein, which had been solubilized separately in the buffer. The pH was maintained at 9.0 with NaOH and finally adjusted to 7.0 with HCl to stop the reaction. All the palmitoyl protein solution was then exhaustively dialyzed at 4 C against distilled water maintained at neutrality and concentrated. A control was run where the reaction pH was adjusted to 7.0 and dialysis started immediately.

When the mole ratio of 16:0-Osu to lysine was kept low and the reaction time kept constant at 60 min, bulk preparations were possible having very low degrees of incorporation as shown in Figure 1, which shows the extent of incorporation at different mole ratios of 16:0-Osu to lysine as compared to the loss of available lysine. It is apparent that under the reaction conditions a maximum of about nine lysine residues was reactive although 11 moles of 16:0 were introduced. It was confirmed by Pronase-E digestion

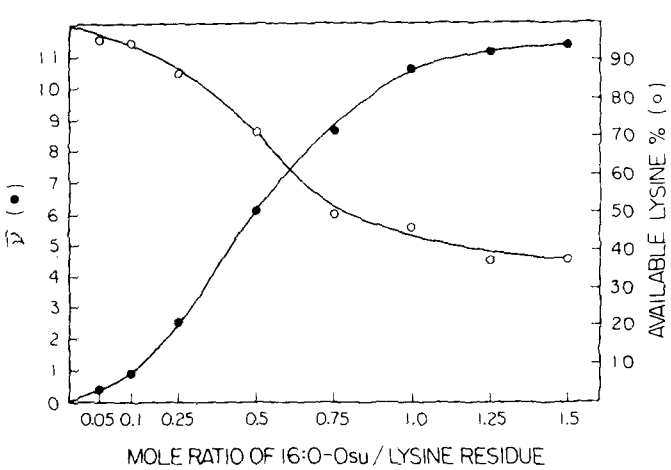

FIG. 1. Increase in incorporation, $\bar{\nu}$, as a function of the mole ratio of N-hydroxysuccinimide ester of palmitic acid (16:0-Osu) to lysine as compared to the available lysine of α_{s1}-casein.

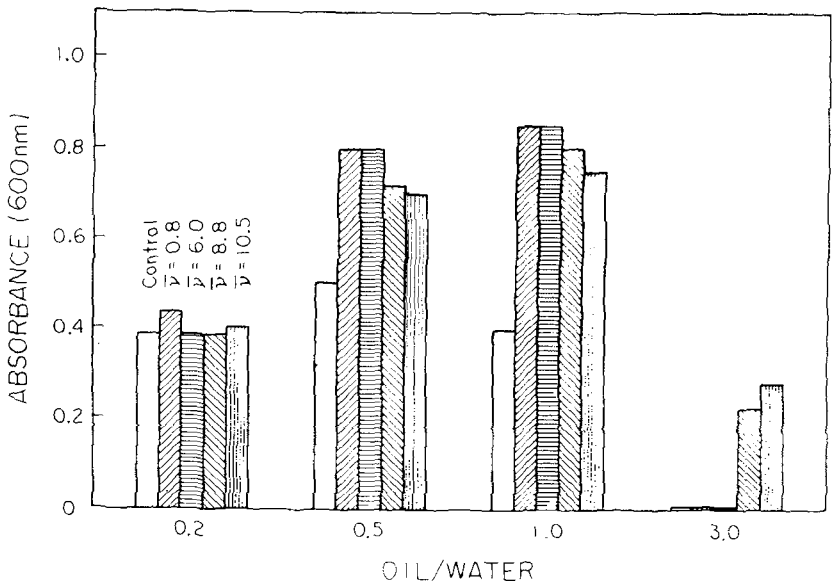

FIG. 2. Comparative emulsifying activity at different oil:water ratios and palmitoyl α_{S1}-casein concentrations. Protein concentrations at each oil:-water ratio (o:w) were as follows: o:w 0.2, 0.33%; o:w 0.5, 0.26%; o:w 1, 0.20%; o:w , 0.10%.

that lysine was predominantly the most reactive residue; tyrosine also reacted; threonine also seemed to react but its reactivity was less. The amino group of lysine was the most reactive nucleophile, and the most susceptible residues appeared to be Lys-34 and/or Lys-36. Tyrosine residues reacted, but the phenolic ester bonds likely were spontaneously cleaved in the alkaline medium of the reaction mixture. Figure 1 shows that the percentage of 16:0-Osu in the reaction mixture that covalently attached to the protein molecule was high when the mole ratio of 16:0-Osu to lysine was 0.25-1.00 and was the highest (85%) when the mole ratio was 0.50, which gave a palmitoyl protein with $\bar{v}$=6 (the average moles of 16:0 covalently bound to one mol of α_{S1}-casein).

Palmitoyl proteins showed an enhanced tendency to associate that was the highest when six ligand molecules were attached, but decreased as the incorporation increased.

Emulsifying activity (EA). Figure 2 shows the EA of the different palmitoyl proteins at different oil:-water ratios. We see that at a ratio of 0.2, control, and all the palmitoyl proteins show similar EA; when the ratio was increased to 0.5, the palmitoyl proteins fared much better, a higher EA being shown by the less incorporated than by the highly incorporated samples. A corollary is that the less incorporated proteins showed higher EA up to an oil:water ratio of 2, but only the highly incorporated proteins showed EA at a ratio of 3.

Emulsion stability (ES). Drainage as a cumulative percentage (CP) of the initial emulsion volume was measured (5) at an oil:water ratio of 0.5. The drainage appeared macroscopically to consist entirely of the aqueous continuous phase. Both the native and control samples showed a high CP of drainage, and the emulsion was completely destabilized within seven days (Table 2). The aqueous drainage of emulsions

TABLE 2.

Drainage as Cumulative Percentage (CP) of Initial Volume for Palmitoyl α_{S1}-Casein Stabilized Emulsions (oil:water ratio of 0.5)

$\bar{v}$	24 hr	48 hr	96 hr	7 days
0 (native)	20.0	24.0	28.0	III[a]
0 (control)	22.2	22.2	24.1	II
0.3	14.8	14.8	14.8	II
0.8	18.5	18.5	18.5	II
2.5	15.4	15.4	15.4	I
6.0	15.4	15.4	15.4	15.4
8.8	16.9	16.9	16.9	16.9
10.5	28.8	28.8	28.8	28.8
11.1	30.7	30.7	30.7	30.7

[a]I, II and III indicate an increasing degreee of estabilization (flocculation and coalescence).

stabilized by the palmitoyl proteins increased as $\bar{v}$ increased. However, the highly incorporated proteins, including that when $\bar{v}$ was 6.0, gave emulsions that were highly stable following the initial drainage. The drainage at an oil:water ratio of one is not shown because the drainage was almost negligible for the palmitoyl proteins, except when $\bar{v}$ was 0.3 it was 9.3% (24 hr), which did not increase any further up to 96 hr but showed distinct signs of coalescence (large oil droplets) after seven days. The highly incorporated samples showed excellent stability; there was no drainage or visible signs of flocculation or coalescence even after seven days, and this was especially noted at an oil:water ratio of 3 when stiff emulsions were obtained.

Fatty acyl peptides. To identify the fatty acylated sites of α_{S1}-casein, fatty acyl peptides (A) were ob-

TABLE 3.

Percentile Distribution of Aggregates of Fatty Acyl Peptide A[a]

Fatty acyl peptide A	Aggregation state				
	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 4.

Amino Acid Composition of Fatty Acyl Peptide A[a]

Amino acids	Amino acids in peptide A (mol/mol)	Mole residues in sequence 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]Amino acid sequence from α_{S1}-casein.

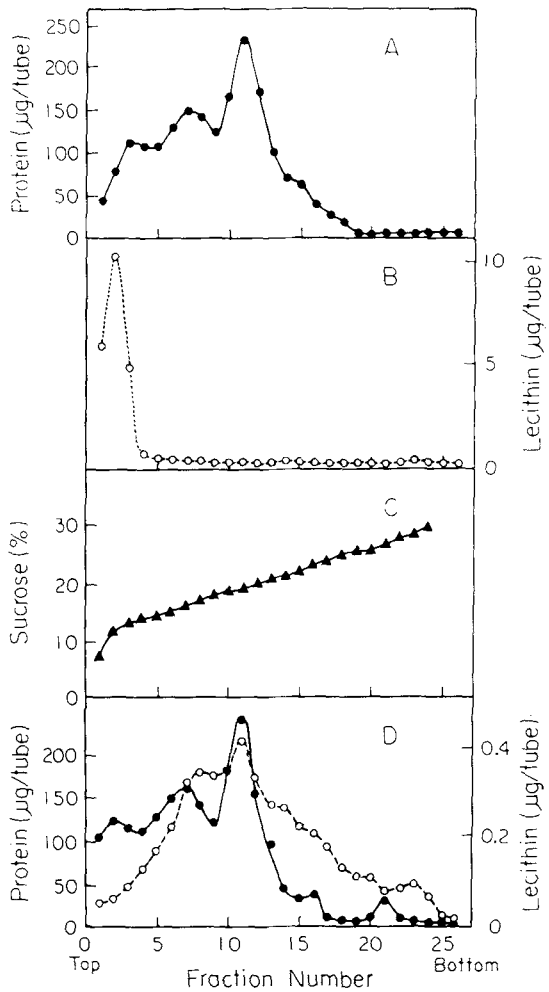

FIG. 3. Formation of the lecithin-soy protein complex. Sucrose density gradient centrifugation was carried out on soy protein —●—, (A); lecithin —○— (B); sucrose (C), and lecithin-protein complex (D).

tained by enzymatic hydrolysis (6) of 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 based on circular dichroism (CD) analysis (6) was essentially the same regardless of the ligand size. Monomer weight was about 3,500, and aggregation numbers determined by pore hydrophilic glass bead column chromatography (6) were 3 (A_3), 6 (A_6), 12 (A_{12}) and 24 (A_{24}); the trimer was the most predominant (Table 3). 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. Table 4 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 ligands 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 -Glu-Asp-Gln. Judging from the primary sequence of α_{S1}-casein (7), 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. Surface tension (dyn/cm) decreased with 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. 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.

ASSOCIATION OF LECITHIN WITH SOY PROTEIN (8-12)

A mixture of radioactive lecithin and defatted soy protein in various molar ratios was weakly sonicated for a short period and analyzed by a linear sucrose density gradient centrifugation (Fig. 3). When the molar ratio of soybean/lecithin was 1:1, various complexes were formed. Major peaks were found at densities 1.07 and 1.08 (fractions 8 and 11). As lecithin was increased to a ratio of 1:5, though a part of

TABLE 5.

Effect of Lecithin on Conformation of Soy Proteins

Lecithin:protein (M:M)	α-Helix (%)		β-Structure (%)		Random coil (%)	
	7s	11s	7s	11s	7s	11s
0:1[a]	100	33	0	6	0	61
0:1	7	5	44	46	49	49
0.6:1	7	4	38	41	55	55
6.3:1	7	4	32	40	61	56

[a]Measured in 2-chloroethanol.

the peak at density 1.07 remained, most of the radioactivity was sedimented downward. Further, as the protein/lecithin ratio increased to 1:25 and 1:100, the major radioactivity floated to density 1.05. The amount of lecithin at density 1.07 at a 1:25 ratio was about 30 times higher than that at a 1:1 ratio. In these experiments, only small amounts of protein and radioactivity were found in the pellet.

Conformation changes of soy proteins by their association with lecithin were determined. In the absence of lecithin, the α-helix content of 7s and 11s proteins was small, while the content was increased in 2-chloroethanol (Table 5). However, lecithin did not affect the α-helix content in these proteins. The β-structure contents in 7s and 11s proteins were 44 and 46%, respectively, and decreased upon their association with lecithin. From the data described above, soy protein and lecithin form definite complexes.

Emulsifying activity [EA]. 7s, 11s And soy protein isolate (SPI) showed a twofold increase in their EA's following the formation of complex with lecithin as compared with the control proteins; ethanol treatment enhanced the EA of the lecithin-protein complex almost fivefold (Fig. 4). Ethanol treatment at a concentration of 40-60% at 25 C for 30 min was most effective in increasing the EA of the lecithin-protein (1:4) complex. However, EA of ethanol-treated 7s, 11s

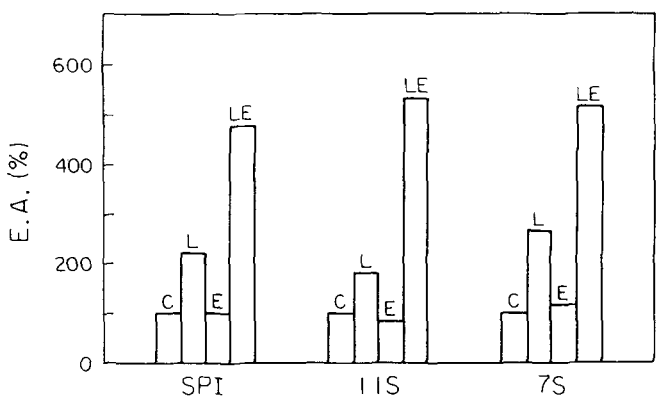

FIG. 4. Effect of ethanol treatment on emulsifying activities of soy protein isolate (SPI), 11s and 7s proteins. C, control; L, lecithin-protein complex; E, ethanol-treated protein; LE, ethanol-treated lecithin-protein complex; EA, emulsifying activity.

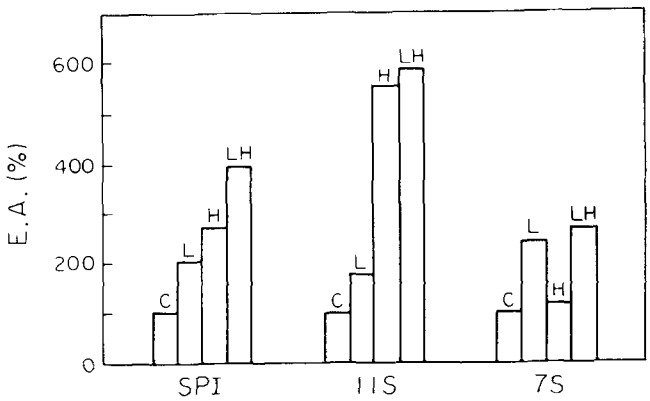

FIG. 5. Effect of heat treatment on emulsifying activity of soy protein isolate (SPI), 7s and 11s proteins. C, control; L, lecithin-protein complex; H, heat-treated protein; LH, heat-treated lecithin-protein complex; EA, emulsifying activity.

and SPI was similar to those of respective control proteins (Fig. 4).

The hardness (viscosity) of emulsions prepared from the spray-dried proteins, soybean oil and water (20:90:100, w/w/w) was measured with a texturometer. The emulsion containing the ethanol-treated lecithin-SPI complex was a creamy paste and showed texturometer readings of 4.1 mm/V, whereas those of the lecithin-SPI complex, ethanol-treated SPI and SPI gave 2.8, 2.0 and 2.0 mm/V, respectively.

It has been reported that heat treatment causes the polymerization of soy protein (13). We examined the effect of heat treatment on lecithin-SPI complexes. When a 4% solution of the lecithin-SPI (1:4) complex was heated in boiling water, EA of the heated lecithin-SPI complex apparently was increased four times as much as that of unheated SPI (Fig. 5). The maximum increase of EA by the heat treatment was lower than that observed with the ethanol treatment. Enhancement of the EA by heating was different from that by the ethanol treatment (Figs. 4 and 5). Heat treatment equally increased EA of 11s and the lecithin-11s protein complex. In other words, this change was caused regardless of whether the lecithin-protein complex was formed. The EA of 7s protein increased by the formation of lecithin-7s protein complex. However, the EA of the lecithin-7s protein complex was not further increased by heat treatment.

To examine conformational changes and molecular sizes of the lecithin-protein complexes following ethanol treatment, CD and gel filtration experiments (12) were carried out. In the region of 210-230 nm, the CD spectrum of the ethanol-treated lecithin-11s protein complex differed from the untreated complex (data not shown). The difference spectrum suggested that the conformational changes were caused by the ethanol treatment. Similar changes were also seen in the case of the lecithin-7s protein complex following ethanol treatment. The untreated lecithin-protein complex of 11s or 7s protein showed gel filtration profiles that were similar to those of the original protein. However, the ethanol treatment caused aggregation of the proteins (data not shown). The elevated EA was found in the aggregated fraction. This indicated

that lecithin and proteins formed a stable complex which could not be separated by gel filtration as described previously (9). Difference CD spectra of the heat treated lecithin-7s protein complex, compared with the untreated sample, indicated few conformational changes caused by heat, in contrast to large changes in conformation of the heated lecithin-11s protein. Such different effects of heat on the conformation of 7s and 11s proteins may reflect the differences in the change of EA (Fig. 5). However, aggregation of both 7s and 11s proteins was caused by heat treatment, as was the case following ethanol treatment.

In order to see whether the increase in EA following ethanol and heat treatments was caused by the same mechanism, the effect of NaCl concentration was examined (12). The increase in EA by ethanol treatment was independent of NaCl concentration, whereas the enhanced effect of heat was reduced by NaCl concentration. From some lines of evidence, it seems likely that ethanol and heat treatments change the conformation of soy protein in different ways. It was also noted that undesirable flavors were removed during the ethanol treatment.

ZEIN-PHOSPHATIDATE COMPLEX

Phosphatidic acid prepared from soy lecithin by phospholipase D reaction associates with the maize protein, zein, to form a zein-phosphatidate complex, which is soluble in water. The complex, in which 7 mol phosphatidate bound to 1 mol zein, shows high emulsifying activity and emulsification capacity in a wide pH range (Table 6). Digestion of the complex by Pronase E increased its emulsification capacity. At pH 7.0, 1 g of the digested complex emulsified 580 ml of soybean oil.

It is concluded that proteins can be endowed with new functional properties by chemical and physical modification. However, physical modification is expected to be more widely used than chemical modification for human foods because of safety concerns.

TABLE 6.

Functional Properties of Zein-Phosphatidate Complex

	Emulsifying activity (%)			Emulsification capacity (ml oil/g)		
	pH 3.0	5.0	7.0	pH 3.0	5.0	7.0
Zein-PA						
intact	0	49	100	0	83	165
Pronase E treatment	85	84	100	165	240	580
Casein	32	0	67	165	0	165
Soy protein	25	0	18	83	0	83

REFERENCES

1. Haque, Z., and M. Kito, *Agric. Biol. Chem. 45*:597 (1982).
2. Haque, Z., T. Matoba and M. Kito, *J. Agric. Food Chem. 30*:481 (1982).
3. Wolf, W.J., and D.R. Briggs, *Arch. Biochem. Biophys. 85*:186 (1959).
4. Haque, Z., and M. Kito, *J. Agric. Food Chem. 31*:1225 (1983).
5. Haque, Z., and M. Kito *Ibid. 31*:1231 (1983).
6. Haque, Z., and M. Kito *Ibid. 32*:1392 (1984).
7. Mercier, J.C., F. Grosclaude and B. Ridadeau-Dumas, *Eur. J. Biochem. 23*:41 (1971).
8. Ohtsuru, M., M. Kito, Y. Takeuchi and S. Ohnishi, *Agric. Biol. Chem. 40*:2261 (1976).
9. Kanamoto, R., M. Ohtsuru and M. Kito, *Ibid. 41*:2021 (1977).
10. Ohtsuru M., Y. Yamashita, R. Kanamoto and M. Kito, *Ibid. 43*:765 (1979).
11. Ohtsuru, M., and M. Kito, *Ibid. 47*:1907 (1983).
12. Hirotsuka, M., H. Taniguchi, H. Narita and M. Kito, *J. Food Sci. 49*:1105 (1984).
13. Mori, T., T. Nakamura, and S. Utsumi, *Ibid. 47*:26 (1981).

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