Specific Limited Hydrolysis and Phosphorylation of Food Proteins for Improvement of Functional and Nutritional Properties

Jean-Marc Chobert[1], Mahmoud Sitohy[2] and John R. Whitaker*
Department of Food Science and Technology, University of California, Davis, CA 95616

Limited specific hydrolysis of casein by *Staphylococcus aureus* V8 protease was used to produce 2% and 6.7% hydrolysates (2 and 6.7% of the peptide bonds hydrolyzed), each containing five polypeptides (by gel filtration) ranging in size from ~16,000 to ~1,000 daltons. The mixtures of polypeptides had substantially increased solubilities at pH 4.0-4.5, near the isoelectric point of casein. In general, the emulsifying activity index was less for the hydrolysates than for casein; the emulsion stability was higher for the 2% hydrolysate than was the emulsion from casein. Phosphorylation of zein markedly increased the water solubility of zein above and below pH 4. When the free amino acids tryptophan and/or lysine were added to zein in the presence of $POCl_3$, some amino acids were covalently bound to zein, in addition to covalent attachment of phosphate groups. Threonine did not become incorporated into zein by this method. These derivatives were much more soluble than zein above and below pH 4, the minimum solubility point. A derivative containing 0.98 mol P/mol of zein, along with 1.05% tryptophan and 0.24% lysine, had a relative growth effect on *Tetrahymena thermophili* of 49% that of casein, in comparison to 4.5% for unmodified zein. All the modified zeins had improved emulsifying activity indices.

The physicochemical and functional properties of proteins can be changed by physical, chemical and enzymatic treatment. These treatments include heating, pH adjustment, hydrolysis and covalent attachment of other constituents.

The purposes of modifying proteins are many. In cooking, the elimination of the toxic effects of proteins such as abrin, ricin, bacterial toxins, protease inhibitors, lectins, etc., is well known. Cooking results in denaturation of proteins, resulting in increased hydrolysis by the digestive enzymes of the gastrointestinal tract, while at the same time increasing or decreasing the solubility of the proteins. Depending on pH of cooking, various chemical modifications of the proteins occur. These include (a) sulfhydryl disulfide rearrangements; (b) oxidation of sulfhydryl groups to disulfide groups; (c) deamidation of the β- and γ-amide groups of asparaginyl and glutaminyl residues; (d) the β-elimination of cysteinyl, cystinyl, phosphorylseryl, phosphorylthreonyl, seryl and threonyl side chains to give dehydroalanyl residues that (e) undergo reaction with nucleophilic groups of proteins such as cysteinyl, lysyl, histidyl, tryptophyl and ornithyl (from hydrolysis of arginyl residues) groups to form intra- and intermolecular cross linking of the protein; (f) racemization of L- to D-amino acid residues in proteins that occurs rapidly above pH 7, the rate being directly proportional to the hydroxide ion concentration, and (g) the well known interaction between glutaminyl and lysyl side chains to give ϵ-(γ-glutamyl) lysyl cross links in proteins on heating (1).

During toasting of peanuts, rice, wheat and baked products and the browning of meats, the amino groups of proteins (primarily ϵ-amino groups of lysyl residues) and the aldehyde and keto groups of reducing sugars form Schiff base intermediates that subsequently result in numerous other products including flavor constituents and melanoids (both desirable). There are also undesirable changes as there is some loss in nutritive quality, and the formation of mutagenic compounds.

pH adjustment is an effective method to modify the properties of proteins, either alone or in conjunction with heating. A few examples will suffice to illustrate this point. The starting point in the production of cottage and brick cheeses is precipitation of the caseins from milk by adjusting the pH, either by fermentation with lactic acid-forming bacteria or by adding acid to near the isoelectric point of these proteins. The solubility of many proteins, including especially those from plants such as soybean, is substantially increased by adjustment of the pH to 11 or 12. The solution then may be formulated into desired products by acidification of the solution in bulk or via extrusion. Gel formation due to three-dimensional protein aggregation as a result of pH adjustment and/or denaturation is a major process in the food industry. Recently, much attention has focused on the mechanisms of gel formation, including the relative importance of hydrophobic interactions, hydrogen bonds, ionic interactions and disulfide bond formation (2–8).

In the last 10 years, the deliberate modification of food proteins in order to change their properties has become increasingly popular as a research area (9–11). The purposes of the modifications are several (12). Modification may be a means of eliminating the undesirable toxic and/or antinutritional properties of some proteins or other constituents; increasing or decreasing the solubility of proteins, especially by adding or eliminating charged groups; changing the functional properties of proteins, which depend in part on changes in solubility of the protein, of gelation, emulsification, and whippability; improving the nutritional properties by the covalent attachment of limiting essential amino acids; and protecting the protein against processing-induced modification, such as the Maillard reaction.

In this paper, we report on changes in the properties of casein as a result of limited proteolysis and of zein as a result of phosphorylation in the absence or presence of limiting essential amino acids.

[1]Present address: Laboratoire des Aliments d'Origine Animale, Institut National de la Recherche Agronomique, La Geraudiere, 44072 Nantes Cedex, France.
[2]Present address: Biochemistry Department, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.
*To whom correspondence should be addressed.

LIMITED PROTEOLYSIS

The use of hydrolysis of proteins in the food processing industry to change their functional properties is well known. Most hydrolyses involve proteases and the peptide bonds. Included among these uses are products requiring extensive hydrolysis, such as vegetable bouillons and malting and chillproofing of beer (14). Processes based around more limited proteolysis include tenderization of meats; coagulation of proteins for the production of cheese and cheese-like products (15, 16); solubilization of protein concentrates (see next paragraph); protein modification in doughs (17); the removal of undesirable flavors, pigments and toxic compounds (15); and modification of the functional properties of proteins.

The relative insolubility, due to native structure, extraction conditions or denaturation, of some of the plant and animal proteins limits their usefulness in food products. Research has shown that the solubility and/or extractability of proteins of beans (18), rapeseed concentrate (19), alfalfa meal (20), heat denatured cottonseed meal (21) and coconut meal (22) and fish protein concentrate (23) can be enhanced by proteolysis.

Three quite different types of controlled-size protein hydrolysate are used commercially. The first type involves essentially complete hydrolysis of plant proteins for production of soy sauce (24, 25), bouillons, stock for soups and for intravenous feeding. The second involves the plastein-type reactions (15). The third, the topic of this section, involves controlled proteolysis to produce polypeptides of narrow molecular weight range between 3,000 and 10,000.

The advantages of controlled-size polypeptides include lack of bitterness (usually), pH-independent solubility and enhanced functional properties. Partial hydrolysis of soy proteins (3.2–8.3% hydrolysis of peptide bonds) increased the solubility at the isoelectric point by 5–20 times that of the original protein and rendered the solubility independent of pH (26). The product was useful for fortification of soft drinks and fruit juices. The emulsifying capacity of partial hydrolysates [DH (degree of hydrolysis) = 1–8%] of soy proteins produced by Alcalase treatment was somewhat less than that of the original protein, while the whipping expansion was markedly enhanced by Alcalase treatment (some 12X), but less so with Neutrase (3–4X) (26).

Gunther (27) reported marked improvement of the foam volume and foam stability of protease-treated soy proteins. In a study of more extensively (DH = 50–70%) hydrolyzed 7S and 11S fractions of soy protein by Alcalase and pronase, Kang (28) reported that the solubility at the isoelectric point (pH 5) was increased several times over that of the original protein but was actually somewhat lower at pH 6. The heat coagulation properties and foam expansion of the hydrolysates were somewhat higher than the control, but the calcium precipitation, emulsifying and foaming stability properties were less than the control (original protein).

A key to the successful commercialization of controlled-size polypeptides is control of proteolysis to give a uniform product. Attempts to achieve this control include: stopping the reaction after reaching a desired level of proteolysis [measured by pH stat (26), by the 2,4,6-trinitrobenzenesulfonate method (29) or by the ninhydrin method]; the use of continuous ultra- and hyperfiltration during proteolysis (30); and the proposed use of highly specific proteases (13).

The remainder of this section addresses the question of whether a highly specific protease can be used to produce controlled-size polypeptides from casein with improved solubility and functional properties.

Specific partial hydrolysis of casein by Staphylococcus aureus *V8 protease. S. aureus* V8 protease specifically cleaves peptide bonds of proteins on the carboxy terminal side of glutamyl residues (31,32). Based on the glutamyl composition and percentage of each of the types of casein (α_{s1}-, α_{s2}-, β- and κ-) in Hammarstein casein, maximum hydrolysis of whole casein expected would be 10.1%. The percentage hydrolysis of casein at various times by *S. aureus* V8 protease is shown in Table 1.

TABLE 1.

Hydrolysis of Casein by *Staphylococcus aureus* V8 Protease[a]

Time (min)	Hydrolysis[b] (%)	Time (min)	Hydrolysis (%)	Time (hr)	Hydrolysis (%)
1	1.20	30	2.59	2	4.42
5	1.59	40	2.61	3	4.77
10	1.78	50	2.78	6	5.11
15	2.06	60	3.07	23	5.63
20	2.28			48	6.71

[a]7.5% casein in H_2O), 400 units enzyme/mM casein, pH adjusted to 7.8, 45 C. MW of casein, 23,000.
[b]Hydrolysis was determined by the 2,4,6-trinitrobenzenesulfonate method of Adler-Nissen (33).

Size distribution of polypeptides. Further studies were done on casein and casein hydrolysates with 2.0 and 6.7% hydrolysis (Fig. 1).

The number and size of the peptides formed were determined by size-exclusion chromatography in a Superose 12 FPLC Pharmacia column (1.5 x 30 cm) using a buffer containing 6 *M* urea and 0.1% 2-mercaptoethanol in a 0.05 *M* sodium phosphate-0.15 *M* NaCl-0.02 *M* EDTA buffer at pH 7.0 (Fig. 1). The column was calibrated with a standard protein mixture containing ferritin, bovine serum albumin (dimer and monomer), ovalbumin, β-lactoglobulin, myoglobin, α-lactalbumin and cytochrome c. The 2% hydrolysate contained five polypeptides of molecular size ~16,000 (30% of total), ~9,000 (26%), ~6,000 (13%), ~3,000 (23%) and ~1,000 (6%). The 6.7% hydrolysate contained five polypeptides of molecular size ~14,000 (11%), ~9,000 (31%), ~3,000 (32%), ~2,000 (15%) and ~1,000 (9%). These results by size-exclusion chromatography were in agreement with results obtained by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate, 6 *M* urea and 0.05% 2-mercaptoethanol in Tris borate buffer, pH 8.0 (data not shown).

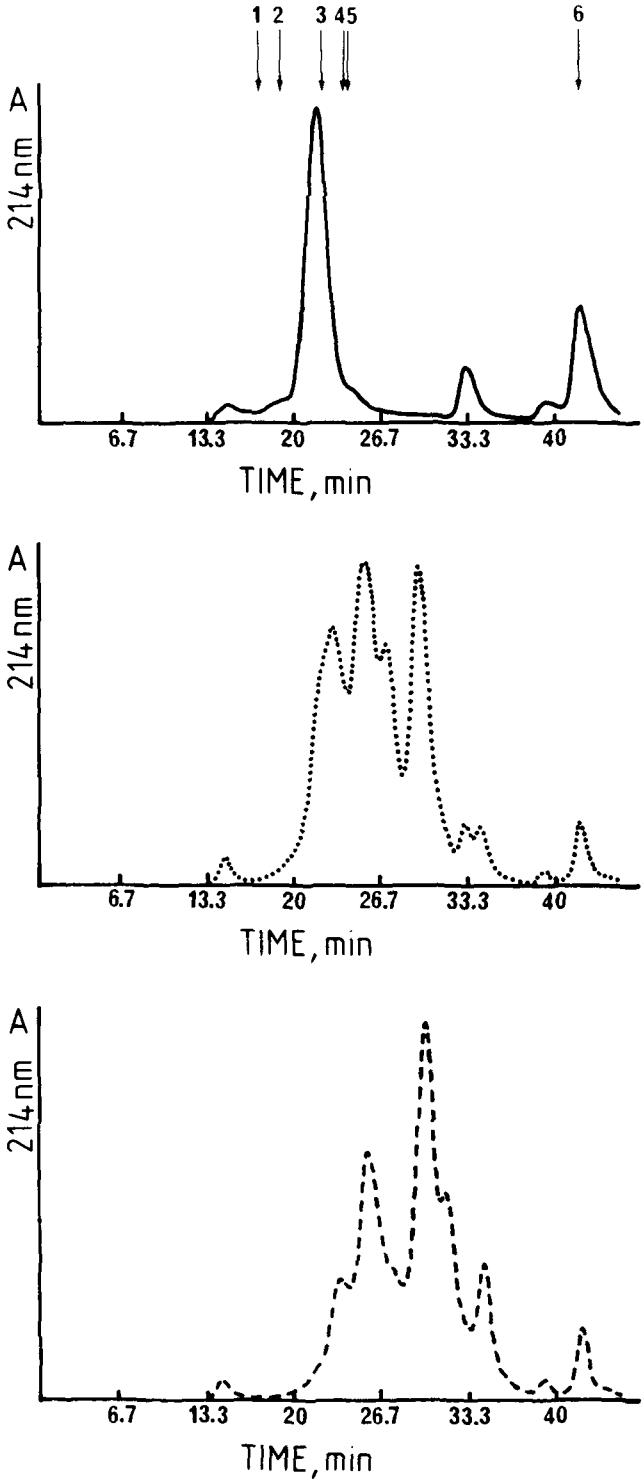

FIG. 1. Elution profiles of casein and enzymatically produced peptides. Separation was by size-exclusion chromatography in a Superose 12 FPLC Pharmacia column (1.5 x 30 cm) equilibrated and eluted with 0.05 *M* phosphate buffer, pH 7.0, containing 6 *M* urea, 0.15 *M* NaCl, 0.02 *M* EDTA and 0.1% mercaptoethanol. Chromatograms from top to bottom are: control casein (———); sample Cn2 (2% hydrolysate; ••••); sample Cn6 (6.7% hydrolysate; – – – – –). The arrows indicate elution times of standard proteins and 2-mercaptoethanol: 1, bovine serum albumin, 67,000 MW; 2, ovalbumin, 43,000 MW; 3, β-lactoglobulin, 18,200 MW; 4, α-lactalbumin, 14,400 MW; 5, cytochrome c, 12,400 MW; 6, 2-mercaptoethanol, 78 MW).

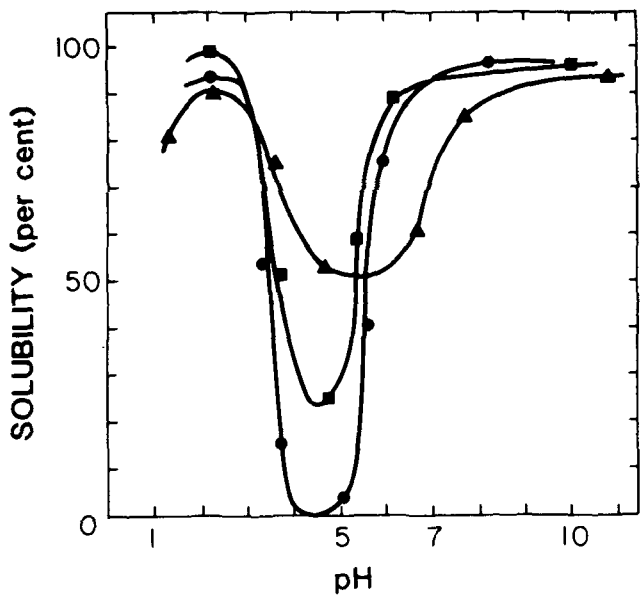

FIG. 2. Solubility of control casein and of *S. aureus* V8 protease-modified casein as a function of pH. Solubility was measured after a 30-min equilibration period at 23 C. The solubility is expressed as percent of total protein (0.1%) in solution. Symbols used: ●, casein; ■, 2% hydrolysate; ▲, 6.7% hydrolysate.

Solubilities of casein and hydrolysates. The solubilities of casein and the two hydrolysates were compared over a wide pH range (Fig. 2).

For solubility determinations, the samples were dissolved in water (0.1% sample, w/w) at 23 C by mixing gently with a Vortex mixer. The pH was adjusted from 1.0 to 11.0 by using HCl or NaOH of high concentration to prevent dilution. After 30 min equilibration at 23 C, the samples were centrifuged at 1640 *g* and 23 C for 15 min, and the protein content of the supernatant was determined by the method of Lowry et al. (34), using bovine serum albumin as a standard. The solubility was expressed as a percentage of the original 0.1% protein solution.

The polypeptides produced by partial hydrolysis of casein had increased solubility near the isoelectric point of casein (pH 4.0-4.5). The solubility of 0.1% casein was essentially zero at pH 4.0-4.5, the 2% hydrolysate had 24% solubility and the 6.7% hydrolysate had 53% solubility. The solubility of the hydrolysates was slightly less at pH 2 than the casein (91, 94 and 100% for 6.7, 2 and 0% hydrolysate, respectively). At pH 7.0, the casein and 2% hydrolysate had equal solubility (93%), while the 6.7% hydrolysate had 74% solubility. The minimum solubility of the 6.7% hydrolysate was at pH 5.5, somewhat higher than for the casein and 2% hydrolysate, with the entire curve shifted to higher pH with respect to casein. This was not the case for the 2% hydrolysate.

Emulsifying activity and stability. The emulsifying activity index (E.A.I.) for casein and the two hydrolysates was compared from pH 1 to 11 (Fig. 3). The E.A.I. was determined by the spectroturbidity method of Pearce and Kinsella (35), with slight modification. The emulsions were prepared by combining

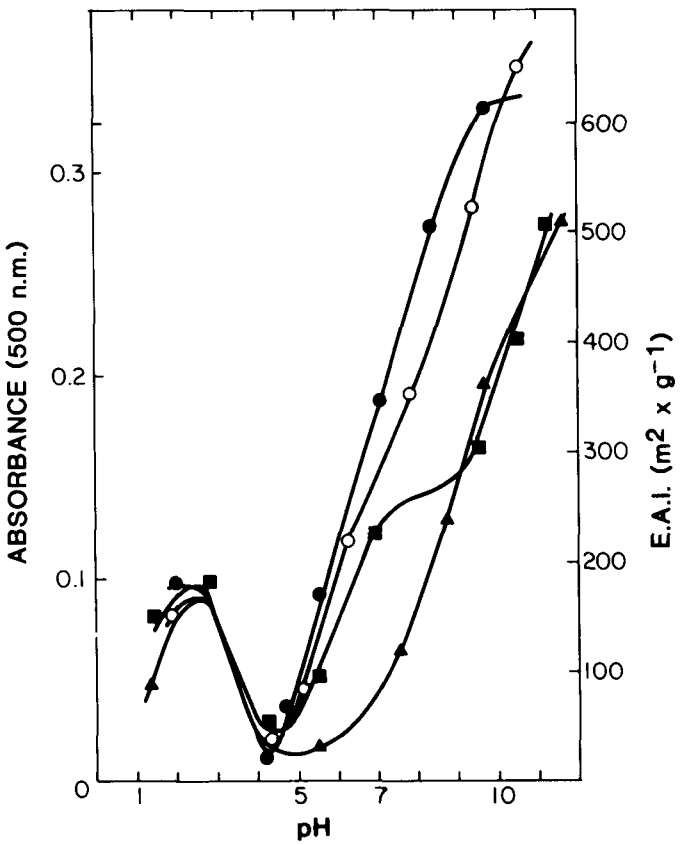

FIG. 3. Turbidity and emulsifying activity index (E.A.I.) of casein, 2% hydrolysate and 6.7% hydrolysate as a function of pH. Symbols used: •, casein; ○, casein heated for 30 min at 100 C; ■, 2% hydrolysate; ▲, 6.7% hydrolysate. E.A.I. = 2T/∅c, where T = turbidity = 2.3A/ (A = absorbance at 500 nm; ℓ = light path in m), ∅ = oil phase volume (0.25 in these experiments) and c = concentration in weight of protein material before emulsion is formed (0.1%).

21 ml of 0.1% casein solution and 7 ml of soybean oil, adjusting the pH to between 1 and 11 and homogenizing the mixture in a stainless micro-container with a Waring blender at 21,000 rpm for 30 sec at 23 C. Aliquots (0.5 ml) of the emulsion were serially diluted (1/1000 final dilution) with 0.1% (w/w) sodium dodecyl sulfate in 0.1 *M* NaCl, pH 7.0. The absorbance at 500 nm was measured immediately.

As shown in Figure 3, E.A.I. of casein increased at pHs above and below pH 4.0-4.5. The increase in E.A.I. was much more at pHs above 4.5 than below pH 4.0. Thermal treatment of casein (30 min at 100 C) had little effect on E.A.I. below pH 6. Above pH 6.0, heat treatment decreased E.A.I. With the 2% hydrolysate, all the emulsions broke in the pH range of 3.5-5.5. From pH 5.5 to 7.0, E.A.I. was appreciably lower than the control casein. At pH 7.0 and above, E.A.I. was similar to that of the 6.7% hydrolysate. With the 6.7% hydrolysate, no stable emulsion was obtained in the pH range 3.5-7.0. At pH 7.5 and above, E.A.I. was much lower than that of casein and heat-treated casein.

The emulsion stability was determined after 24 hr at 23 C, as described above for E.A.I. The E.A.I. was also determined after heating the 24-hr sample at 80 C for 30 min. Emulsifying stability was calculated by the formula

$$\Delta E.A.I.\% = \frac{E.A.I._{max} - E.A.I._{80\,C}}{E.A.I._{max}} \times 100$$

where $E.A.I._{max}$ is the maximum value obtained at either t_0 or $t_{24\ hr}$. The results are shown in Table 2. The smaller the value of ΔE.A.I.% the better the stability. Except at pH 6, the 2% hydrolysate had equal or better emulsifying stability than the control casein. The emulsion stability of the 6.7% hydrolysate was less than control casein at all pHs.

The reasons why limited proteolysis of casein by *S. aureus* V8 protease gave polypeptides with poorer emulsifying properties than did limited proteolysis of soy protein (26,27) are not clear. Casein is generally more soluble than soy protein over the pH range used and casein has emulsifying properties superior to those of soy protein. Addition of a glutamic acid residue at the carboxyl end of each polypeptide may have adversely affected emulsifying properties. Another possibility is that there are not enough amphiphilic polypeptides formed, as required for good emulsifying properties. We are now examining this possibility. Clearly, the specificity of enzyme is of major importance in determining the functional properties of the polypeptides [compare Alcalase and Neutrase in the data of Adler-Nissen and Olsen (26)].

TABLE 2.

Emulsion Stability of Control and Protease-Modified Caseins[a]

pH	Control casein	Hydrolysates	
		2%	6.7%
2	6	1	11-34
3	7	0	12-45
6	5	14	n.d.[b]
8	0	0	3-15
9	1	2	3-20
10	4	3	3-22
11	10	2-4	8-15

[a]Results are expressed as % of the difference between E.A.I. maximum value (after 24 hr at 20 C) and that after thermal processing (for 30 min at 80 C) (see text).
[b]n.d., Not determined because of emulsion collapse.

INCREASED SOLUBILITY OF PROTEINS BY COVALENT ATTACHMENT OF HYDROPHILIC GROUPS

The solubility of proteins can be improved by limited hydrolysis as discussed in the previous section or by increasing the number of hydrophilic groups of proteins. Increase in the number of hydrophilic groups of a protein could be accomplished by converting glutaminyl (Gln) and asparaginyl (Asn) residues to glutamyl (Glu) and aspartyl (Asp) residues by deamidation, or by covalent attachment of hydrophilic amino acid residues, glyco groups or phosphate groups.

We are not aware of any systematic research that has been done on the enzymatic conversion of Gln and Asn to Glu and Asp in food proteins. Replacement of neutral amino acid residues with acidic amino acid residues would be expected to have a major effect on the properties of proteins from wheat and other cereals, where up to one-third of the total amino acids is Gln. Deamidation can be accomplished at pH 8 to 9 at elevated temperatures. Unfortunately, some racemization and β-elimination are also likely to occur. Needed is an enzyme with broad enough specificity to deamidate a substantial number of the groups.

Hydrophilic amino acids have been covalently attached to proteins. Puigserver et al. (36) attached various amounts of aspartyl groups to casein by use of the active N-hydroxysuccinimide ester method. The most extensively modified casein contained 10 additional aspartyl groups/mol of casein. The modified protein was 85% as soluble as casein at pH 5.5 and 4 C. The viscosity of the modified protein was 1.05 times that of casein, and the UV spectrum indicated a difference in tertiary structure between the two proteins.

Glyco groups have been covalently attached to proteins by several methods (37-40). Marshall and Rabinowitz (38) attached cyanogen bromide-activated dextran to trypsin, α-amylase and β-amylase. They found the conjugates to be more heat stable and less susceptible to proteolysis than the unconjugated proteins. Krantz et al. (39) attached thioglycosides to proteins by amidination, diazocoupling and amide formation. Gray (37) and Lee et al. (40) attached reducing sugars to proteins by a modification of the reductive alkylation method described by Means and Feeney (41). Lee et al. (40) reported that casein modified by covalent attachment of glucose, fructose or lactose had lower in vitro digestibility by α-chymotrypsin and lower nutritive value in rat feeding experiments than casein. The carbohydrate-protein conjugates were reported to be soluble, but none of the workers above reported any quantitative data on solubility.

Phosphorylation of proteins has been achieved with a variety of chemical reactions (42-44). In most cases, the intent was to add a single residue of phosphate to the protein. In experiments designed to attach several phosphate groups, Woo et al. (45) and Matheis et al. (42) used phosphorus oxychloride to add several phosphates to β-lactoglobulin, casein and lysozyme. Sung et al. (46) reported the successful phosphorylation of soy protein with trisodium trimetaphosphate; however, Matheis et al. (42) were unable to reproduce the work. It is difficult to remove all the adsorbed phosphate from the proteins, thereby showing the phosphate to be covalently bound. Matheis and Whitaker (43) also covalently attached phosphate groups to proteins by reductive alkylation with glyceraldehyde 3-phosphate. Phosphorylated casein and phosphorylated lysozyme adsorbed more water at 43% relative humidity and 25 C than did control casein (42); however, decreased water solubility was reported (42,47), possibly as a result of cross linking of the protein.

Solubility and nutritional improvement of zein: Phosphorylation of zein. Zein was dissolved in 80% acetone-20% water to give a 5% solution. $POCl_3$ was dissolved in CCl_4 to give a 20% solution. The cooled $POCl_3$ solution was added drop by drop to the protein solution in an ice bath. The temperature was maintained between 0-20 C during the reaction and the pH between 5-8 by the addition of 50% NaOH as needed. Reaction time was 30 min to 2 hr depending on the ratio of $POCl_3$ to protein used. The phosphorylated zein, insoluble in 80% acetone, was filtered on a Whatman #1 filter paper and the remaining acetone removed by evaporation at 23 C under a hood. The dry powder was dissolved in water, the pH adjusted to 7 and the solution dialyzed against 0.1 *M* KCl for one day at 4 C with several changes and then against deionized water for five days with several changes. The solution was treated with a mixed bed resin (Bio-Rad AG 501-X8) for 3 hr at 23 C, with the pH maintained at pH 7 by addition of 5 *N* NaOH. The supernatant was removed and concentrated by ultrafiltration on Diaflo membrane PM 10. The concentrated solution was lyophilized.

Many different preparations of phosphorylated zein, in the absence and presence of some limiting essential amino acids, were prepared. Some representative results are reported here.

Composition and nutritional quality of modified zeins. Total phosphorus was determined by the method of Harwood et al. (48) after sample digestion with perchloric acid. Free inorganic phosphorus was determined as described by Matheis et al. (42). Protein was determined by the method of Lowry et al. (34) using bovine serum albumin as a reference protein. The amino acid composition was determined using the method of Hamilton (49) on a Phoenix Automated Amino Acid Analyzer. Tryptophan was determined by the base-hydrolysis method of Concon (50). The solubility and functional properties were determined as described for modified and unmodified caseins in the first part of this paper, except the emulsions were diluted to a final value of 1/500. The nutritional quality of the derivatives was determined using the *Tetrahymena thermophili* bioassay method described by Matheis et al. (42).

Analytical data on zein and the seven modifications of zein are reported in Table 3. Samples A, D and 6 were phosphorylated only. The importance of both zein concentration and the ratio of $POCl_3$ to protein on the extent of phosphorylation is indicated by samples A, D and 6. Varying the zein concentration from 1.20 to 4.75% while keeping the ratio of $POCl_3$ constant increased the extent of phosphorylation 9.6 times (samples D and A). Keeping the zein concentration essentially constant but increasing the ratio of $POCl_3$ to protein four times increased the extent of phosphorylation 3.7 times (samples A and 6). At 5% zein and 800 and 1,600 molar ratio of $POCl_3$ to zein, 20 and 40 mol P/mol zein were bound, respectively (data not shown). The relative growth rate of *T. thermophili* was very low on zein, compared to casein, and even lower on the phosphorylated samples because of further reduction in tryptophan and lysine content, and also methionine (not shown).

Tryptophan and lysine were covalently bound to

TABLE 3.

Phosphate, Tryptophan and Lysine Content and Relative Growth Rate of *Tetrahymena thermophili* on Modified Zeins

Sample	Zein concn. (%)	Molar ratio[a] ($POCl_3$/zein)	P bound (mol P/mol zein)	Amino acid bound[b]		Relative Growth[c]
				Trp	Lys	
Casein	—	—	—	—	—	100
Zein	—	—	0	0.16	0.09	4.5
A	4.75	100	2.60	0.09	0.00	1.1
D	1.20	100	0.27	0.09	0.00	1.1
6	5.00	400	9.5	—	—	—
T10	5.00	200	1.07	0.61(10)[d]	0.09	22.2
L40	5.00	200	1.20	0.09	0.46(40)[d]	8.8
M1	5.00	200	0.98	1.05(5)[d]	0.24(10)[d]	48.8
Mr1	5.00	400	12.3	0.63(5)[d]	0.22(10)[d]	24.4

[a]Molecular weight of zein, 38,000.
[b]Total, in percentage, including small amounts in the original zein.
[c]Growth relative to that on casein.
[d]Molar ratio of free amino acid to zein added to the reaction mixture.

zein when the free amino acids were added into the mixture of zein and $POCl_3$ (Table 3). Tryptophan and lysine are the most limiting essential amino acids in zein, being nearly absent (Table 3). Threonine, the third limiting essential amino acid in zein, was not covalently bound to zein over a range of concentrations (data not shown).

Phosphorylation of zein in the presence of a tenfold molar ratio of tryptophan to protein increased the tryptophan content to 0.61% (sample T10). The relative growth rate of *T. thermophili* on this sample was 22.2% that of casein. Use of a thirtyfold molar ratio of tryptophan to zein gave 0.86% tryptophan in zein (data not shown).

Addition of a fortyfold molar ratio of lysine to zein in the presence of $POCl_3$ gave 0.46% lysine in the product (sample L40). The relative growth rate of *T. thermophili* on this sample was 8.8%, indicating that for *T. thermophili* the most limiting essential amino acid in zein is tryptophan. Increasing the ratio of lysine to zein to 80 did not improve the extent of incorporation.

Tryptophan and lysine added together at molar ratios to zein of 5 and 10, respectively, resulted in 1.05% tryptophan and 0.24% lysine in the product (sample M1) (Table 3). The relative growth of *T. thermophili* on the modified zein was 48.8% that on casein, a 10.8-fold improvement over that of the original zein. Addition of a higher ratio of $POCl_3$ to zein (sample Mr1) resulted in a significantly higher level of phosphorylation, as expected from results on samples A, D and 6. However, the level of tryptophan incorporation was lower than when a 200-fold ratio of $POCl_3$ to zein was used (sample M1), resulting in a product that was less effective in supporting growth of *T. thermophili.*

The mechanism by which $POCl_3$ activates the covalent incorporation of amino acids into proteins is not known. To our knowledge, this is the first time this reaction has been reported. One could postulate that the amino acid is incorporated either through forming an activated derivative directly with the amino acid, such as a mixed phosphate-carboxyl anhydride, or by the amino group of the amino acid acting as a nucleophile to displace phosphoesters from the phosphorylated protein. The locations of the covalent attachment of the phosphate groups and the amino acids have not been determined.

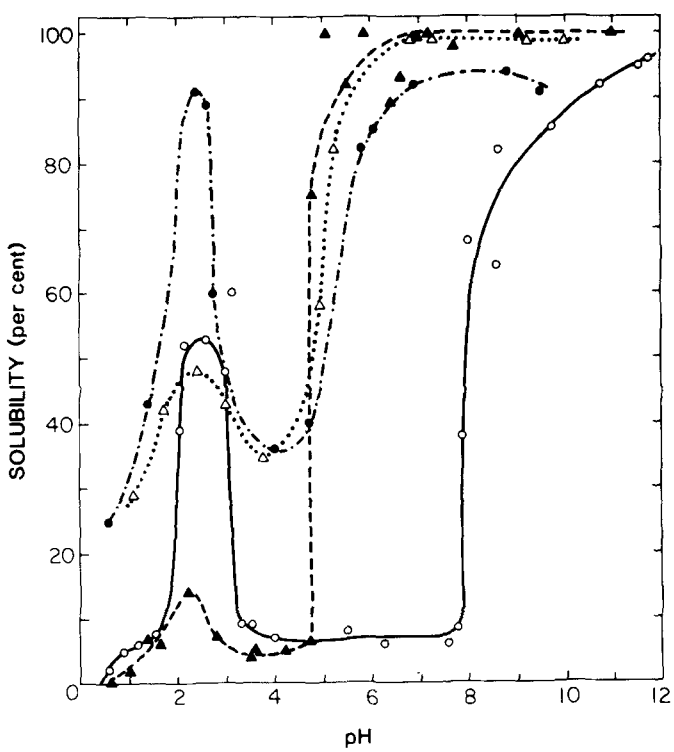

FIG. 4. pH-Solubility profiles for zein and for phosphorylated zeins. Solubility was determined with 0.1% suspensions of protein at 23 C. ○—○, zein; ●—•—●, sample D (Table 3) containing 0.27 mol P/mol zein; △•••△, sample A (Table 3) containing 2.60 mol P/mol zein; ▲———▲, sample 6 (Table 3) containing 9.5 mol P/mol zein.

Solubility of zein and modified zeins. Zein is not very soluble in water below pH 1.5 and between 3.3 and 7.8 (Fig. 4). At pH 2.5, ~50% of a 0.1% solution of zein is soluble; the solubility increases above pH 7.7 to almost complete solubility at pH 11.7. Phosphorylation of zein changes the pH-solubility curve markedly. The minimum solubility of the three phosphorylated zeins is at pH 4.0. Between pH 4.6 and 7.7, the phosphorylated samples have appreciable solubility, unlike the original zein. Above pH 4.6, the solubility is higher the higher the extent of phosphorylation. However, in the region of pH 2.5, the solubility is in reverse order of the extent of phosphorylation.

The covalent attachment of phosphate and amino acids to zein also drastically changes the pH-solubility profile of zein (Fig. 5). The solubility minimum of the derivatives is at pH 4.0-4.3. Solubility is increased markedly between pH 4.6 and 7.5, in contrast to the original zein. The extent of phosphorylation of samples T10, L40 and M1 is similar (Table 3), yet there is considerable difference in solubilities of the three samples. On the alkaline side of the solubility minimum, at pH 6, solubility increases in the order of samples L40, M1 and T10. On the acid side of the solubility minimum, at pH 2.5, all three derivatives are more soluble than the original zein.

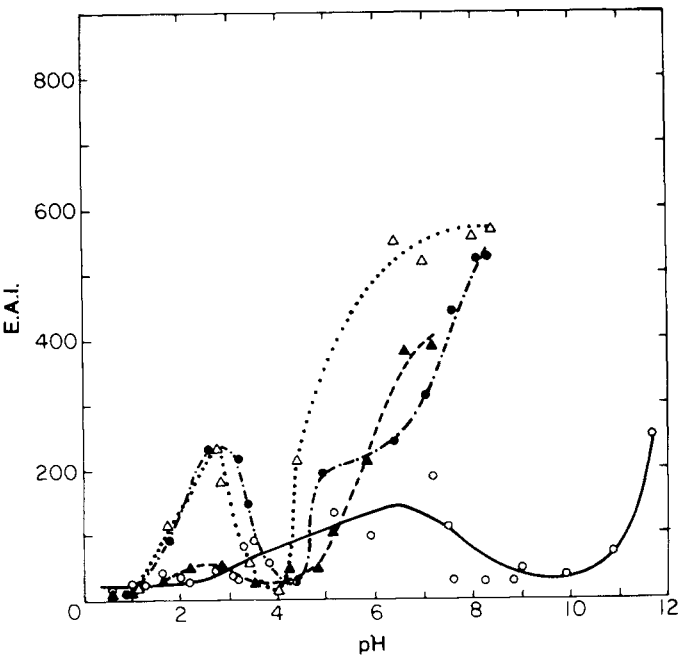

FIG. 6. pH-Emulsifying activity index profiles for zein and for phosphorylated zeins. The emulsifying activity index was determined on 0.1% protein suspensions at 23 C as described in the text. ○—○, zein; ●—•—●, sample D (Table 3) containing 0.27 mol P/mol zein; △•••△, sample A (Table 3) containing 2.60 mol P/mol zein; ▲———▲, sample 6 (Table 3) containing 9.5 mol P/mol zein.

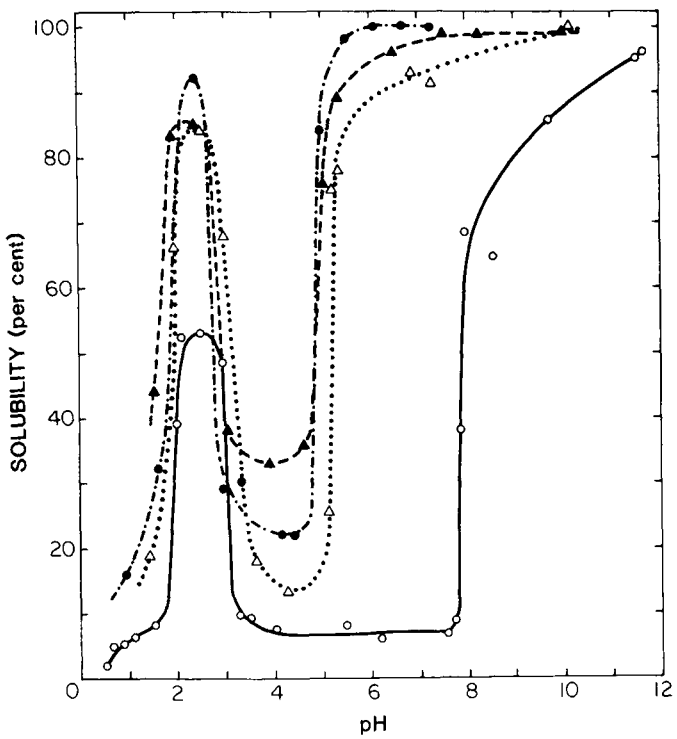

FIG. 5. pH-Solubility profiles for zein and for zein containing covalently attached phosphate and tryptophan and/or lysine. Solubility was determined with 0.1% suspensions of protein at 23 C. ○—○, zein; ●—•—●, sample T10 (Table 3) containing 1.07 mol P/mol zein and 0.61% tryptophan; △•••△, sample L40 (Table 3) containing 1.20 mol P/mol zein and 0.46% lysine; ▲———▲, sample M1 (Table 3) containing 0.98 mol P/mol zein, 1.05% tryptophan and 0.24% lysine.

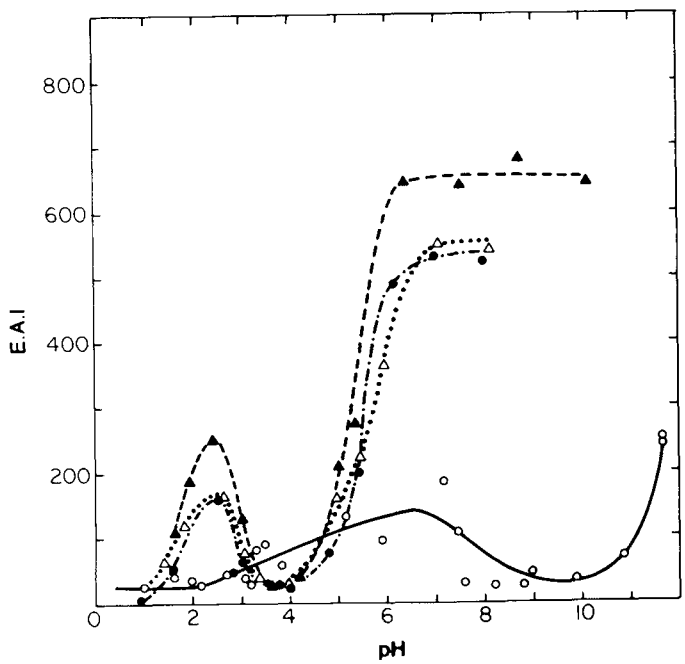

FIG. 7. pH-Emulsifying activity index profiles for zein and for zein containing covalently attached phosphate and tryptophan and/or lysine. The emulsifying activity index was determined on 0.1% protein suspensions at 23 C as described in the text. ○—○, zein; ●—•—●, sample T10 (Table 3) containing 1.07 mol P/mol zein and 0.61% tryptophan; △•••△, sample L40 (Table 3) containing 1.20 mol P/mol zein and 0.46% lysine; ▲———▲, sample M1 (Table 3) containing 0.98 mol P/mol zein, 1.05% tryptophan and 0.24% lysine.

Emulsifying properties of zein and modified zeins. The emulsifying activity indices (E.A.I.) for zein and the modified zeins are shown in Figures 6 and 7. Zein is relatively inactive as an emulsifier from pH 0.6 to 11, with a small broad increase in E.A.I. around pH 6.3. All of the derivatives have higher E.A.I. values than the original zein except around pH 4.0. The E.A.I. curves approximately mirror the solubility of these derivatives (Figs. 4 and 5). There is a smaller peak of emulsifying activity at pH 2.5 and then a much higher increase in emulsifying activity above pH 5. Above pH 5, the derivative with 2.60 mol P/mol zein (sample A) has a higher E.A.I. than either the derivative with 0.27 or 9.5 mol P/mol zein (samples D and 6). At pH 2.5, sample 6, with the highest level of phosphorylation, has the lowest E.A.I.

Samples T10, L40 and M1 have similar pH-E.A.I. profiles (Fig. 7). On both sides of the minimum, sample M1, with both tryptophan and lysine attached, has the highest E.A.I., with samples L40 and T10 being about the same. Comparison of these results with those of Figure 3 for casein shows that the modified zein is as effective as casein as an emulsifying agent. In contrast to the results with the partially hydrolyzed casein products, there is little doubt that the increase in solubility produced by modification of zein parallels its effectiveness as an emulsifying agent.

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REFERENCES

1. Bjarnason, J., and K.J. Carpenter, *Br. J. Nutr. 24*:313 (1970).
2. Hermansson, A.-M., *J. Texture Stud. 9*:33 (1978).
3. Saio, K., and T. Watanabe, *Ibid. 9*:135 (1978).
4. Kinsella, J.E., *J. Am. Oil Chem. Soc. 56*:242 (1979).
5. Shimada, K., and S. Matsushita, *Agric. Biol. Chem. 44*:647 (1980).
6. Shimada, K., and S. Matsushita, *J. Agric. Food Chem. 29*:15 (1981).
7. Peng, I.C., D.W. Quass, W.R. Dayton and C.E. Allen, *Cereal Chem. 61*:480 (1984).
8. Mori, T., T. Nakamura and S. Utsumi, *J. Agric. Food Chem. 34*:33 (1986).
9. *Food Proteins. Improvement Through Chemical and Enzymatic Modification, Adv. Chem. Ser. 160,* edited by R.E. Feeney and J.R. Whitaker, American Chemical Society, Washington, D.C., 1977, 312 pp.
10. *Modification of Proteins: Food, Nutritional and Pharmacological Aspects, Adv. Chem. Ser. 198,* edited by R.E. Feeney and J.R. Whitaker, American Chemical Society, Washington, D.C., 1982, 402 pp.
11. *Protein Tailoring for Food and Medical Uses,* edited by R.E. Feeney and J.R. Whitaker, Marcel Dekker, New York, 1985, 392 pp.
12. Feeney, R.E., and J.R. Whitaker, in *New Protein Foods, Vol. 5,* edited by H.L. Wilcke and A.M. Altschul, Academic Press, New York, 1985, p. 181.
13. Whitaker, J.R., and A.J. Puigserver, *Adv. Chem. Ser. 198*:57 (1982).
14. Whitaker, J.R., in *Enzymes. The Interface Between Technology and Economics,* edited by J.P. Danehy and B. Wolnak, Marcel Dekker, New York, 1978, p. 53.
15. Fujimaki, M., S. Arai and M. Yamashita, *Adv. Chem. Ser. 160*:156 (1977).
16. Whitaker, J.R., *Food Technol. 32*:175 (1978).
17. Beuchat, L.R., *Cereal Chem. 54*:405 (1977).
18. Sreekantiah, K.R., H. Ebine, T. Ohta and M. Nakano, *Food Technol. 23*:1055 (1969).
19. Hermansson, A.-M, D. Olsson and B. Holmberg, *Lebensm.-Wiss. Technol. 7*:176 (1974).
20. Childs, E.A., J.L. Forte and Y. Ku, *ACS Symp. Ser. 47*:304 (1977).
21. Childs, E.A., *J. Food Sci. 40*:78 (1975).
22. Molina, M.R., and P.A. LaChance, *J. Food Sci. 38*:607 (1973).
23. Hevia, P., J.R. Whitaker and H.S. Olcott, *J. Agric. Food Chem. 24*:383 (1976).
24. Fukushima, D., *Food Rev. Int. 1*:149 (1985).
25. Yong, F.M., and B.J.B. Wood, *Adv. Appl. Microbiol. 17*:157 (1974).
26. Adler-Nissen, J., and H.S. Olsen, *ACS Symp. Ser. 92*:125 (1979).
27. Gunther, R.C., *J. Am. Oil Chem. Soc. 56*:345 (1979).
28. Kang, Y.-J., Ph.D. thesis, Graduate School of National Fisheries, University of Pusan, Korea, 1985.
29. Fields, R., *Methods Enzymol. 25B*:464 (1972).
30. Olson, H.S., and J. Adler-Nissen, *ACS Symp. Ser. 154*:133 (1981).
31. Drapeau, G.R., Y. Boily and J. Houmard, *J. Biol. Chem. 247*:6720 (1972).
32. Houmard, J., and G.R. Drapeau, *Proc. Nat. Acad. Sci. U.S.A. 69*:3506 (1972).
33. Adler-Nissen, J., *J. Agric. Food Chem. 27*:1256 (1979).
34. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.S. Randall, *J. Biol. Chem. 193*:265 (1951).
35. Pearce, K.N., and J.E. Kinsella, *J. Agric. Food Chem. 26*:716 (1978).
36. Puigserver, A.J., L.C. Sen, E. Gonzales-Flores, R.E. Feeney and J.R. Whitaker, *J. Agric. Food Chem. 27*:1098 (1979).
37. Gray, G.R., *Arch. Biochem. Biophys. 163*:426 (1974).
38. Marshall, J.J., and M.L. Rabinowitz, *Ibid. 167*:777 (1975).
39. Krantz, M.J., N.A. Holtzman, C.P. Stowell and Y.C. Lee, *Biochemistry 15*:3963 (1976).
40. Lee, H.S., L.C. Sen, A.J. Clifford, J.R. Whitaker and R.E. Feeney, *J. Agric. Food Chem. 27*:1094 (1979).
41. Means, G.E., and R.E. Feeney, *Biochemistry 7*:2192 (1968).
42. Matheis, G., M.H. Penner, R.E. Feeney and J.R. Whitaker, *J. Agric Food Chem. 31*:379 (1983).
43. Matheis, G., and J.R. Whitaker, *Ibid. 32*:699 (1984).
44. Matheis, G., and J.R. Whitaker, *Int. J. Biochem. 16*:867 (1984).
45. Woo, S.L., L.K. Creamer and T. Richardson, *J. Agric. Food Chem. 30*:65 (1982).
46. Sung, H.-Y., H.-J. Chen, T.-Y. Liu and J.-C. Su, *J. Food Sci. 48*:716 (1983).
47. Salak, J., Z. Vodraza and J. Cejka, *Collect. Czech. Chem. Commun. 30*:1036 (1965).
48. Harwood, J.E., R.A. van Steenderen and A.L. Kuhn, *Water Res. 3*:417 (1969).
49. Hamilton, P.B., *Anal. Chem. 35*:2055 (1963).
50. Concon, J.M., *Anal. Biochem. 67*:206 (1975).

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