Solubility and Emulsifying Properties of Caseins Modified Enzymatically by *Staphylococcus aureus* V8 Protease

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Casein was treated with *Staphylococcus aureus* V8 protease to give 2 and 6.7% hydrolysis of the peptide bonds. *S. aureus* V8 protease has specificity only for peptide bonds in which glutamic acid contributes the carbonyl group. After 2% hydrolysis, 70% of the resulting peptides had molecular weights below 12 400, as determined by size-exclusion chromatography. After 6.7% hydrolysis, 90% of the peptides had molecular weights below 12 400. The solubility of the hydrolysates was much higher than that of casein at pH 4.0–5.0 (0, 25, and 50% for control casein, 2% hydrolysate, and 6.7% hydrolysate, respectively). The emulsifying activity of the hydrolysates was reduced over that of casein at all pHs.

Modification of a protein usually refers to physical, chemical, or enzymatic treatments changing its conformation, its structure, and consequently its physicochemical and functional properties. This subject has been widely reviewed (Kinsella, 1976; Cheftel, 1977; Friedman, 1977, 1979; Feeney and Whitaker, 1977; Kinsella and Shetty, 1979; Phillips and Beuchat, 1981; Morr, 1982; Whitaker and Puigserver, 1982; Cheftel et al., 1985).

Of the many actual and potential uses of enzymes for modification and improvement of proteins (Whitaker, 1974, 1977), that of hydrolysis of proteins is the most widely used (Fox et al., 1982). Hydrolysis involves the action of selected proteolytic enzymes to split specific peptide bonds in a protein.

The peptides produced by proteolysis have smaller molecular sizes and less secondary structure than proteins and may be expected to have increased solubility near the isoelectric point, decreased viscosity, and significant changes in the foaming, gelling, and emulsifying properties from those of proteins. The peptides may be useful in various food-processing operations, but very little information has so far been available on the functional properties of peptides produced by proteolysis (Adler-Nissen, 1976, 1984; Adler-Nissen and Olsen, 1979; Olsen and Adler-Nissen, 1979; Gunther, 1979; Adler-Nissen et al., 1983).

Caseins are known to be flexible proteins without a rigid conformation, and their primary structures have been determined (Mercier et al., 1971, 1973; Ribadeau Dumas et al., 1972; Brignon et al., 1977). Furthermore, caseins have amphiphilic properties. Studies using caseins and peptide derivatives may provide valuable information on the relationships between protein structure and functional properties.

Enzymatically hydrolyzed caseins are used in candy manufacture (Fox, 1970). Kumetat and Beeby (1954) reported that pronounced hydrolysis of milk proteins, to a polypeptide content of 5–40%, produced a product that could be substituted for egg proteins for meringue. Haggett (1974) reported that proteolysis improved solubility of lactic acid precipitated casein. A limited degree of hydrolysis of whey proteins with pepsin improved the emulsifying and foaming capacities, whereas hydrolysis with Pronase, under similar conditions, caused excessive hydrolysis and loss of these functions (Kuehler and Stine, 1974). Shimizu et al. (1983) have determined the emulsifying properties of bovine α_{s1}-casein and its peptides that were formed by limited proteolysis with pepsin or papain. Recently, solubilization of heat-denatured whey proteins has been achieved by hydrolysis with papain and protease A2 (a serine protease from *Bacillus licheniformis*) (Saint-Paul et al., 1984).

Peptide size control is essential if optimum and reproducible changes in functional properties are to be achieved. Various workers have attempted to accomplish this by control of time–temperature–enzyme relations, by following the rate of peptide bond hydrolysis (Adler-Nissen and Olsen, 1979) and by removal of peptides by continuous ultra- and hyperfiltration during proteolysis (Olsen and Adler-Nissen, 1981). This control of size might be accomplished more readily by use of highly specific proteases (Whitaker and Puigserver, 1982).

In this paper, we describe the changes in solubility and emulsifying activity of bovine casein digested with *Staphylococcus aureus* V8 protease, which specifically cleaves peptide bonds on the COOH terminal side of glutamic acid (Drapeau et al., 1972; Houmard and Drapeau, 1972).

MATERIALS AND METHODS

Materials. Casein, as a purified powder, was obtained from Sigma Chemical Co., St. Louis, MO. *S. aureus* V8 protease (570 units/mg of material at pH 7.8 with casein as substrate) was obtained from Miles Laboratories, Elkhart, IN. One unit of enzyme is that amount of enzyme required to cause a change in extinction at 280 nm of 0.001 AU/min under the conditions of assay. Sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid, and L-leucine were obtained from Sigma Chemical Co. Soybean oil (partially hydrogenated soybean oil and polyglycerides made from vegetable oils) was obtained from Hunt-Wesson Foods, Inc., Fullerton, CA. Electrophoresis calibration kit was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents and chemicals were of analytical grade.

Limited Proteolysis of Casein with *S. aureus* V8 Protease. Preliminary experiments were conducted to determine the best conditions of hydrolysis. These experiments included investigation of (1) ratio of enzyme to substrate (E/S = 2.2, 4.4, 8.7, 13.0, 17.4, or 22 units of enzyme/g of casein); (2) influence of substrate concentration ([S] = 1.0, 2.5, 5.0, or 7.5% (w/v) at an enzyme concentration of 0.8 unit of enzyme/mL); (3) influence of

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temperature (T = 37, 45, 50, or 55 °C at 17.4 units of enzyme/g of casein).

The following conditions were selected for the results reported here. A 7.2% (w/v) stock casein solution was prepared in distilled water by adjusting to pH 7.80 with 1 N NaOH and stirring until dissolved. The reaction mixture contained 0.8 unit of enzyme/mL and 0.046 g of casein/mL (E/S = 17.4 units of enzyme/g of casein). The final concentration of casein was 4.6%. The mixture was incubated at 45 °C and rocked back and forth during the reaction. At various times, 0.5-mL aliquots of the hydrolysate were diluted with SDS (1% final concentration) and heated 15 min at 100 °C to stop the reaction. The solutions were then lyophilized.

The degree of hydrolysis (DH) was determined spectrophotometrically by the trinitrobenzenesulfonic acid method according to Adler-Nissen (1979). On the basis of the glutamyl residue composition of the major caseins, the maximum degree of hydrolysis of peptide bonds expected with *S. aureus* V8 protease was about 10%.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out on gel slabs (8.2 cm × 13.9 cm × 2.7 mm) (12% acrylamide, 0.12% *N,N'*-methylenebis(acrylamide)) containing 0.1% SDS and 0.1% 2-mercaptoethanol, with or without 6 M urea. The gel buffer was 0.2 M Tris, 0.33 M boric acid, and 0 or 6 M urea at pH 8.8. The electrode buffer was 0.03 M Tris, 0.05 M boric acid, pH 8.0, containing 0.1% SDS, 0 or 6 M urea, and 0.05% 2-mercaptoethanol. The sample buffer was 0.03 M Tris, 0.05 M boric acid, pH 8.3, containing 4% SDS, 0 or 6 M urea, and 0.1% 2-mercaptoethanol. The samples were boiled in the buffer for 2 min and cooled prior to application to the gel slab. Electrophoresis was carried out at 10 mA/gel, at 4 °C for 8 h. Proteins were stained with 0.2% Coomassie Brilliant Blue R-250 in 5% acetic acid–30% ethanol. The gels were destained with 5% acetic acid–30% ethanol.

Size-Exclusion Chromatography. Size-exclusion chromatography was performed in a Superose 12 FPLC Pharmacia column (1.5 × 30 cm). The sample was dissolved in the buffer and filtered on a Sartorius 0.22-μm filter before application to the column. The eluant was 50 mM phosphate buffer, pH 7.0, containing 0.15 M NaCl and 0.02 M EDTA. The flow rate was 0.5 mL/min. The effluent was monitored at 214 nm. The area of each peak was determined by a Shimadzu CR3A recorder–integrator. Another fractionation was performed in the same buffer, but with 6 M urea and 0.1% 2-mercaptoethanol.

The column was calibrated with a standard protein mixture containing ferritin (MW 444 000), dimer BSA (134 000), BSA (67 000), ovalbumin (43 000), β-lactoglobulin (18 300), myoglobin (17 800), α-lactalbumin (14 400) and cytochrome c (12 400).

Solubility. Enzyme-treated casein and control caseins were dispersed in distilled water (0.1%, w/w) by mixing with a Vortex. The pH was adjusted from 1.0 to 11.0 with HCl or NaOH of high normality to limit dilution. A part of each solution was used to determine emulsifying activity; the rest was centrifuged for 15 min at room temperature (International Clinical and Chemical Centrifuge, IEC 428) at a maximum speed of 3400 rpm (RCF = 1640g). After filtration, the protein content of the supernatant was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The solubility was expressed in percentage of total protein concentration.

Emulsifying Activity. To prepare the emulsion, 21 mL of 0.1% casein solution and 7 mL of soybean oil (ϕ (volume fraction of the dispersed phase) = 0.25) were shaken together and homogenized in a stainless-steel microcontainer with a Waring blender, operating at 21 000 rpm for 30 s at room temperature.

With no protein present, the emulsions were unstable and the turbidity varied from experiment to experiment. In a few cases water-in-oil emulsions were formed. The presence of as little as 0.1% protein caused a marked increase in the stability of the emulsion and improved reproducibility. Therefore, all results are reported without correction for turbidity in the absence of protein. The blank was water, which had the same absorbance as 0.1% SDS in 0.1 M NaCl, pH 7.0.

Emulsifying activity of the caseins was evaluated by spectroturbidity according to Pearce and Kinsella (1978), with slight modification. Aliquots were immediately pipetted from the emulsion and diluted 1000-fold into 0.1% (w/v) SDS in 0.10 M NaCl, pH 7.0. The tubes were inverted three times to obtain homogeneous mixtures, and then absorbance at 500 nm was recorded. Identical 1-cm path length glass cuvettes were used and were rinsed with a jet of distilled water and dried between determinations. Absorbance of duplicate aliquots of each emulsion was measured, and the individual values were plotted. The emulsifying activity was expressed as its emulsifying activity index (EAI) EAI = $2T/\phi c$, where T = turbidity = 2.3 A/l (A = absorbance at 500 nm and l = light path in meters), ϕ = oil phase volume = 0.25, and c = concentration of protein (0.1%) before the emulsion is formed.

Pearce and Kinsella (1978) have noted that the measured EAI is a characteristic of the whole system, not of the protein alone since the EAI value is affected by the type of emulsifying apparatus used, the speed of the apparatus and time used, the amount and type of oil used, and the total emulsion volume.

Emulsion Stability. The stock emulsions prepared above were held at room temperature for 24 h. After stirring, aliquots were diluted and turbidity was measured as described above (EAI, 20 °C). The 24-h-old emulsions were then heated at 80 °C for 30 min. After cooling to room temperature and stirring, turbidity was again measured as above (EAI, 80 °C). The emulsion stability was calculated by

$$\Delta\text{EAI}\% = \frac{\text{EAI}_{\max} - \text{EAI}_{80°\text{C}}}{\text{EAI}_{\max}} \times 100$$

where $\text{EAI}_{\max}$ is the maximum value obtained either at t_0 or t_{24h}. These values did not differ significantly. The smaller the value of ΔEAI%, the better the stability.

Samples of control casein heated at 100 °C for 30 min were included in these experiments.

RESULTS AND DISCUSSION

Limited Proteolysis of Casein with *S. aureus* V8 Protease. After 1 h of incubation of casein with enzyme, the further rate of hydrolysis was quite slow (Figure 1). After 3, 6, 24, and 48 h, the degrees of hydrolysis (DH) were 4.85, 5.1, 5.6, and 6.7%, respectively. Hydrolysates at two times were selected to determine solubility and emulsifying activity of the peptide mixtures: a 2% DH (Cn2) and a 6.7% DH (Cn6) obtained after 15-min and 48-h hydrolysis, respectively.

Results of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE results of control and enzyme-treated casein in the presence of SDS and 2-mercaptoethanol (with or without 6 M urea) are shown in Figure 2. Separation is based primarily on size. A plot of log molecular weight vs mobility by using the reference proteins (Figure 2Ad, 2Ba) provided some

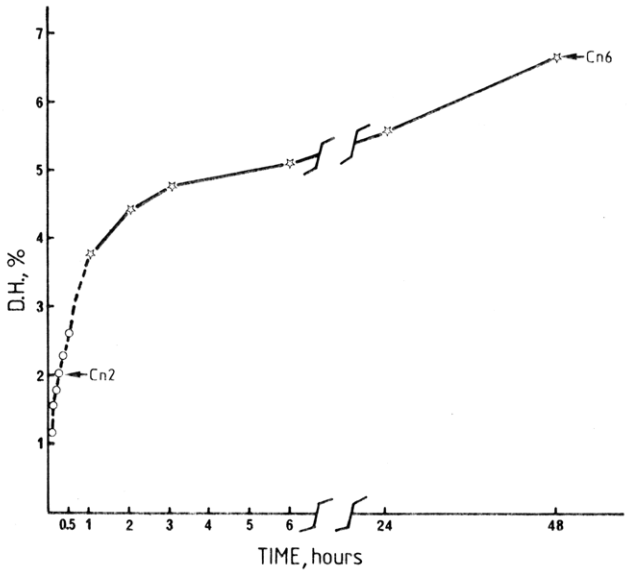

Figure 1. Rate of hydrolysis of casein by *S. aureus* V8 protease. Reaction conditions: 4.6% casein, E/S = 17.4 units of enzyme/g of casein, pH 7.80 (no added buffer ions), and 45.0 °C. Cn2 (○) and Cn6 (☆) identify samples removed at 15 min and 48 h, respectively.

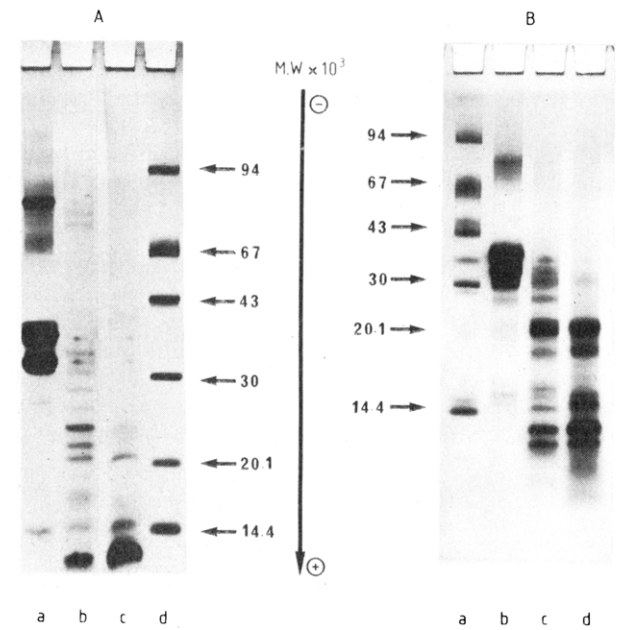

Figure 2. SDS-PAGE of casein and enzymatically produced peptides from casein. Conditions as in Materials and Methods in absence (A) and presence (B) of 6 M urea. A: a, control casein; b, sample Cn2; c, sample Cn6; d, reference proteins (top to bottom) phosphorylase *b* (92 000/subunit), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), Kunitz soybean trypsin inhibitor (21 000), and α-lactalbumin (14 400). B: a, reference proteins (see A); b, control casein; c, sample Cn2; d, sample Cn6.

information on the size of peptides formed after hydrolysis. In the absence of enzyme treatment and 6 M urea (Figure 2A), casein separated into three major bands (α- and β-caseins of MW 33 000–40 000; κ-casein of MW ~80 000). Caseins are known to form associating systems in the absence of urea (Yaguchi and Tarassuk, 1967). After 15-min enzyme treatment (Cn2), 16 bands of various intensities of dye staining were seen (Figure 2Ab). The major band had MW <14 400 (~11 500–12 000) with three other prominent bands of MW ~20 000–24 000. After 48 h of hydrolysis (Cn6; Figure 2Ac), the majority of the product

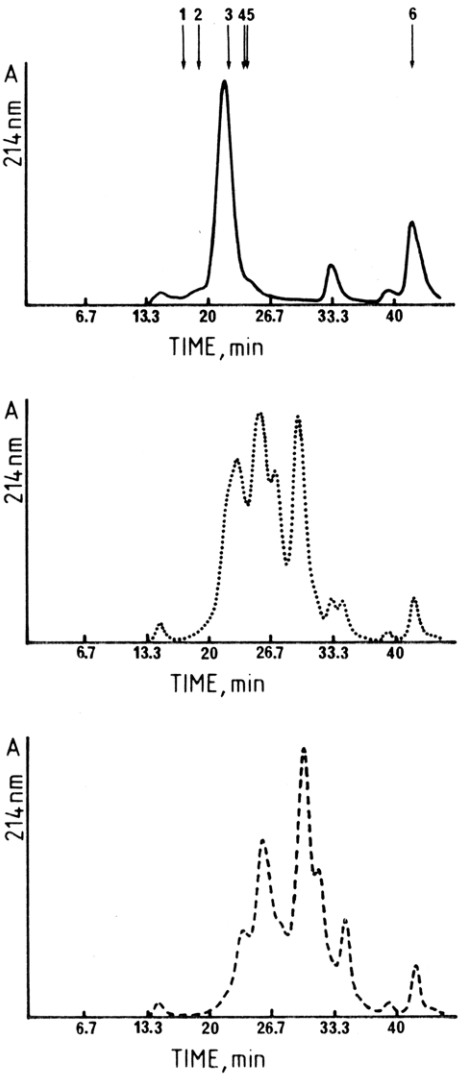

Figure 3. Elution profiles of casein and enzymatically produced peptides. Separation was by size-exclusion chromatography in a Superose 12 FPLC Pharmacia column (1.5 × 30 cm) equilibrated and eluted with 0.05 M phosphate buffer, pH 7.0, containing 6 M urea, 0.15 M NaCl, 0.02 M EDTA, and 0.1% mercaptoethanol.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

had MW <14 400 with smaller bands at ~14 000 and ~21 000. Better resolution of the caseins and the peptides was obtained in the presence of 6 M urea (Figure 2B). The control caseins had MW in the range of ~35 000, with a smaller band near 80 000 (Figure 2Bb). After 15 min of enzyme treatment (Cn2; Figure 2Bc), at least 11 bands were visible with the three major bands at MW ~23 000–25 000 and <14 400 (~10 000–11 000). Small amounts of the original caseins were still detectable. After 48 h of enzyme treatment (Cn6; Figure 2Bd), at least six bands were present with apparent MW ~24 000, 18 000, 14 000–15 000, and <14 400 (~10 000).

Size-Exclusion Chromatography. Size-exclusion chromatography was used to verify the SDS-PAGE results and to obtain more quantitative data on the size distribution (Figure 3). Integration of the areas under the peaks indicated that after a 2% DH, 70% of the resulting peptides had molecular weights below 12 500. After a 6.7%

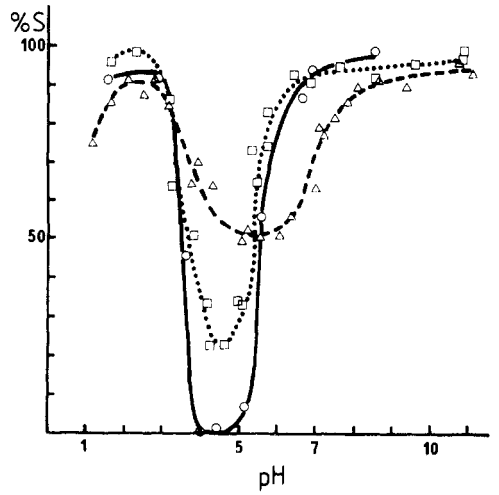

Figure 4. Solubility of control and protease-modified caseins as a function of pH. Solubility was measured after a 30-min equilibration period at room temperature (23 °C). The solubility is expressed as percent total protein (0.1%) in solution. Symbols: ○, control casein; □, sample Cn2; △, sample Cn6.

DH, 90% of the resulting peptides had molecular weights below 12 500.

Solubility. After partial hydrolysis (6.7% DH) of casein with *S. aureus* V8 protease, the peptide solution had a minimum solubility at pH 5.0–6.0, in contrast to pH 4.0–5.0 for the control casein (Figure 4). The solubility of the peptides was much higher than that of the control casein in the range pH 4.0–5.1. Between pH 5.5 and 9–10 the solubility of the peptides was lower than that of the control casein.

With a 2% and a 6.7% DH, solubility in the p*I* (isoelectric point) range increased from 0 to 25 and 50%, respectively. Therefore, one may conclude that the more hydrolyzed the casein, the more soluble it is near the p*I*.

Previously, it was shown that a 30-min heating at 50 or 80 °C did not seem to modify the solubility of casein (Chobert et al., 1987). So, the increases in solubility observed after enzymatic digestion are only due to hydrolysis.

Emulsifying Activity. Emulsions were prepared from casein solutions over a range of pH values. The emulsifying activity index (EAI) increased at pHs above and below pH 4.0–4.5 (Figure 5). The increase in EAI was much more pronounced at values higher than pH 5.5, as compared to that below pH 4.5. The minimum at pH 4.0–4.5 is presumably because of flocculation due to minimum protein solubility. This pH corresponds to the isoelectric point of the caseins.

Thermal treatment of casein for 30 min at 100 °C had little effect on EAI below pH 6. However, above pH 6.0, heat treatment decreased EAI somewhat.

After protease treatment for 15 min (2% DH), all the emulsions broke in the range pH 3.5–5.5. From pH 5.5 to 7.0, the EAI was somewhat lower than the casein control. Between pH 7.0 and 9.5, the EAI showed a marked shift to that typical of the more extensively hydrolyzed sample.

After protease treatment for 48 h (6.7% DH), no emulsion was obtained in the range pH 3.5–7.5. The base-line absorbance is not 0, since the oil–protein–water system had a small absorbance in relation to the blank. Between pH 2 and 10, the emulsifying activity was lower than that observed for the control or heated casein.

Emulsifying Stability. After 24-h storage and heating of the emulsion, a large decrease of emulsifying activity index between pH 2 and 10 was observed for sample Cn6, which gave quite variable results. Under the same conditions, EAI of control casein decreased about 5–10% and

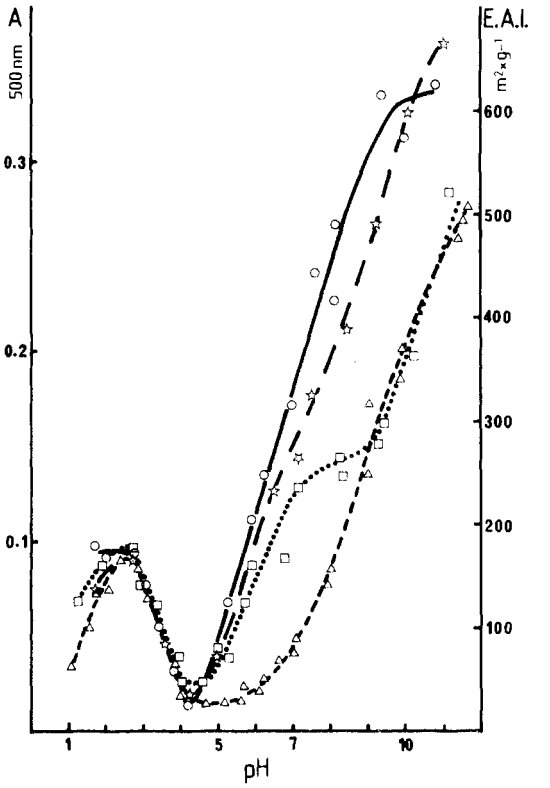

Figure 5. Absorbance at 500 nm and emulsifying activity index (EAI) of control and protease-modified caseins as a function of pH. See Materials and Methods for conditions. Symbols: ○, control casein; ☆, control casein heated for 30 min at 100 °C; □, sample Cn2; △, sample Cn6.

Table I. Emulsion Stability of Control and Protease-Modified Caseins after 0 and 24 h at 20 °C and 30 min at 80 °C[a]

pH	control casein	Cn2	Cn6
2	6	1	22
3	7	0	28
6	5	14	nd[b]
8	0	0	9
9	1	2	12
10	4	3	12
11	10	2–4	12

[a] Results are expressed as percent of the difference between EAI_{max} value and that after thermal processing (see the Experimental Section). [b] nd = not determined because of emulsion collapse.

EAI of sample Cn2 was more stable, showing a 0–5% decrease in general, except for a 14% decrease at pH 6.0 (Table I).

During the formation of an emulsion under ideal conditions, soluble protein diffuses to and concentrates at the oil–water interface once the interfacial electrostatic barrier is overcome. Solubility of protein is an important prerequisite for film formation because rapid migration to and adsorption at the interface is critical. After partial proteolysis of casein with *S. aureus* V8 protease, solubility was increased particularly in the p*I* range, with 0%, 25%, and 50% solubility for control casein, Cn2, and Cn6, respectively. In the pH range around the p*I*, it was not possible to form an emulsion. These results near the p*I* show that increasing the solubility did not increase the emulsifying activity. The decrease in emulsifying activity above the p*I* observed on enzyme treatment seems to indicate that the smaller peptides were not able to form a stable film surrounding the fat globules.

Adler-Nissen and Olsen (1979) reported that 3–8% hydrolysis of the peptide bonds of soy proteins with Alcalase at pH 8.0 increased the solubility of the product at pH 5.0 by 5–20 times over that of the original soy protein. The solubility of the product was essentially pH independent, making it useful for inclusion in highly acidic fruit drinks and other acid foods. The limited proteolysis had little effect on the emulsifying capacity of the soy protein, but there was a very substantial increase (12-fold at 3% hydrolysis) in the whipping expansion of the product. After 3% hydrolysis of the same soy protein with Neutrase, the whipping expansion was only 4-fold larger than the untreated protein. Therefore, the specificity of the protease is quite important.

Gunther (1979) reported a marked effect of protease treatment on comparative whip rates of soy proteins. For example, after 10 min of whipping, the density of the soy protein isolate was 0.75 while that of the protease-treated soy protein isolate was 0.30. The stability of the foam from protease-treated soy protein, with whipping for up to 7 min, was close to that of chicken egg white; however, continued whipping led to collapse of the foam.

An explanation of why the peptides produced from limited hydrolysis of casein with *S. aureus* V8 protease had decreased emulsifying activity is not known. An examination of the primary structure of the caseins shows the glutamyl residues to be nearly uniformly spaced throughout the molecules. Perhaps the presence of a highly negatively charged glutamyl residue at the carboxyl end of the chain adversely affects the emulsifying properties. Perhaps the majority of the peptides are not amphiphilic, as is needed for good emulsifying properties. Separation and characterization of the peptides would be required to verify this. Clearly, further work is needed in order to predict improvement of functional properties by controlled limited proteolysis of proteins.

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Registry No. *S. aureus* V8 protease, 66676-43-5.

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