Solubility and Emulsifying Properties of Caseins and Whey Proteins Modified Enzymatically by Trypsin

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Casein and whey proteins were treated with trypsin to give, respectively, 4.3, 8.0, 9.9 and 2.5, 3.9, 5.3% hydrolysis of the peptide bonds. For casein hydrolysates, all the resulting peptides had molecular weights below 15000, including 30%, 44%, and 33% between 5000 and 10000 after 4.3, 8.0, and 9.9% hydrolysis, respectively. Of the material, 18% (4.3% hydrolysate) to 66% (9.9% hydrolysate) had molecular weights below 5000, as determined by size-exclusion chromatography. For whey protein hydrolysates, size-exclusion chromatography showed major peaks near 18000, 13000, 9000, and 5000 molecular weights after a 2.5% hydrolysis, near 9000 and 5000 after a 3.9% hydrolysis, and near 6000 and 2000 after a 5.3% hydrolysis. A peak near 1500 was obtained with increasing intensity following increasing hydrolysis. After trypsin treatment, the solubility of the casein hydrolysates was largely increased only at pH 4.0–5.0; however, the solubility of the whey protein hydrolysates was higher than that of control whey protein at all pHs. The emulsifying capacity of casein hydrolysates was increased at the isoelectric point, while with whey protein hydrolysates the emulsifying capacity was increased at alkaline pH. The emulsifying activity of the hydrolysates was higher than that of casein and much higher than that of whey proteins.

Modification of a protein usually refers to physical, chemical, or enzymatic treatments changing its conformation and 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; Kilara and Sharkasi, 1986; Feeney, 1987; Chobert and Mesnier, 1988).

Of the many actual potential uses of enzymes for modifications of proteins and improvement of their functional and nutritional properties (Whitaker, 1974, 1977), that of hydrolysis of proteins is the most widely used (Fox et al., 1982).

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 original 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; Shimizu et al., 1986; Chobert et al., 1988).

Milk proteins, the molecular structure of which being extensively studied, can be used as models for determining some relationships between structure and function.

Primary structures of β-lactoglobulin (Preaux et al., 1979; Braunitzer et al., 1979) and α-lactalbumin (Brew et al., 1970) induce a spacial conformation stabilized by S-S bonds with associations with other proteins or polymerization.

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.

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. Enzymatically hydrolyzed caseins are used in candy manufacture (Fox, 1970). 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). Monti and Jost (1978) showed that the solubility of heat-denaturated whey proteins reached 80 and 100% after Neutrase or papain and trypsin action, respectively. Shimizu et al. (1983, 1986) have determined the emulsifying properties of bovine α_{s1}-casein and its peptides that were formed by limited proteolysis with pepsin or papain. Solubilization of heat-denaturated whey proteins has been achieved by hydrolysis with papain and protease A2 (a serine protease from *Bacillus licheniformis*) (Saint-Paul et al., 1984). Pepsin and polyphosphate have been used for improving whey protein concentrate whippability and gel strength, respectively (To et al., 1985).

Peptide size control is essential if optimum and reproducible changes in functional properties are to be achieved. This control might be accomplished by use of highly specific proteases (Whitaker and Puigserver, 1982). Changes in solubility and emulsifying activity of bovine casein digested with *Staphylococcus aureus* V8 protease have recently been described (Chobert et al., 1988).

In this paper, we describe the changes in solubility and emulsifying properties (emulsifying capacity, emulsifying activity, emulsion stability) of bovine casein and whey protein concentrate digested with trypsin, in relation to the size distribution of the resulting peptides.

MATERIALS AND METHODS

Materials. Casein, as a purified powder, was obtained from Sigma Chemical Co., St. Louis, MO. Whey protein concentrate (WPC), purchased from Protarmor, Cogles, France, was previously ultrafiltered on mineral membranes in order to eliminate lactose and then freeze-dried; the protein content of the lyophilized powder was 80%.

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Trypsin (EC 3.4.21.4.) was purchased from Boehringer, Mannheim, France. Sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid, and L-leucine were obtained from Sigma. Rapeseed oil was from Carrefour, France. Electrophoresis calibration kit was obtained either from Pharmacia, Uppsala, Sweden, or from Sigma. All other reagents and chemicals were of analytical grade.

Limited Proteolysis of Casein and Whey Proteins with Trypsin. Casein (50 g) or ultrafiltered whey protein concentrate (50 g) was dissolved in distilled water (final concentration 5%, w/v) by adjusting to pH 8.0 with 1 N NaOH. Trypsin, previously solubilized in distilled water adjusted to pH 8.0, was added to the reaction mixture in order to give a final E/S ratio from 0.5 to 1%. The mixture was incubated at 37 °C and gently stirred during the reaction. Addition of 0.1 N NaOH was monitored by using a pH-stat (Mettler DL 40 RC). At various times, depending on the degree of hydrolysis to be reached, the enzymatic reaction was stopped either by heating the casein hydrolysate for 30 min at 100 °C or by decreasing the pH of the whey proteins hydrolysate to 1.0; in the latter case, after 60 min, the pH was raised to 8.0. The solutions were then lyophilized.

The degree of hydrolysis (DH) was determined spectrophotometrically by the trinitrobenzenesulfonic acid method according to Adler-Nissen (1979).

A sample of casein (control casein) and whey protein concentrate (control whey proteins) was treated in the same manner but without addition of trypsin, in order to determine the modifications only due to the proteolysis.

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 6 M urea. The gel buffer was 0.2 M Tris, 0.33 M boric acid, and 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, 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, 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 room temperature, for 6 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.

SDS-PAGE was also carried out on gel slabs (17.7 cm × 20 cm × 1.5 mm; 12% acrylamide, 1.2% *N,N'*-methylenebis(acrylamide)) containing 0.1% SDS with 8 M urea. The gel buffer was 1.65 M Tris, 0.9 M phosphoric acid, and 1% SDS at pH 6.8. The gel buffer 10-fold diluted was used as electrode buffer. The sample buffer was 0.09 M phosphoric acid, pH 6.8, containing 1% SDS, 8 M urea, and 1% 2-mercaptoethanol. Electrophoresis, staining, and destaining were carried out as described above.

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 eluant (50 mM phosphate buffer, pH 7.0, containing 6 M urea and 0.15 M NaCl) added with 0.1% 2-mercaptoethanol and filtered on a Sartorius 0.22-μm filter before application to the column. The flow rate was 0.5 mL/min. The effluent was monitored at 280 nm. The area of each peak was determined by a Delsi Enica 10 recorder–integrator.

The column was calibrated with a standard protein mixture containing BSA (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), α-lactalbumin (14 400), and cytochrome *c* (12 400).

Solubility. Control and enzyme-treated casein and WPC 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. After a 30-min equilibration period at room temperature (20 °C), a part of each solution was used to determine emulsifying properties; the rest was centrifuged for 15 min at room temperature (Lab Centrifuge MLW T5) at 5500 rpm. 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 Capacity. Emulsifying capacities of control and enzyme-treated casein and WPC samples were determined according to Webb et al. (1970). In a typical experiment performed at room temperature, 120 mL of a 0.1% protein solution was put in a jar equipped with two electrodes connected to a volt–ohm meter (Multimeter CdA 770) in order to detect the sudden increase in electrical resistance of the dispersion that occurs upon emulsion collapse. Rapeseed oil was delivered with continuous blending (20 000 rpm) at a rate of 60 g/min up to the point of oil inversion in the emulsion. Emulsification capacity was expressed as grams of emulsified oil/sample of protein (120 mg) and as a percentage volume by dividing the oil phase volume required to reach the emulsion breakpoint by the total emulsion volume and multiplying by 100 as suggested by Acton and Saffle (1972).

Emulsifying Activity. To prepare the emulsion, 21 mL of 0.1% protein solution and 7 mL of rapeseed 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 and whey proteins 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 0.1 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 = $2.3A/l$ (A = absorbance at 500 nm and l = light path in meters), Φ = oil phase volume = 0.25, and c is concentration of protein (0.1%) before the emulsion is formed.

Emulsion Stability. The stock emulsions prepared above were held at room temperature (20 °C) 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 the

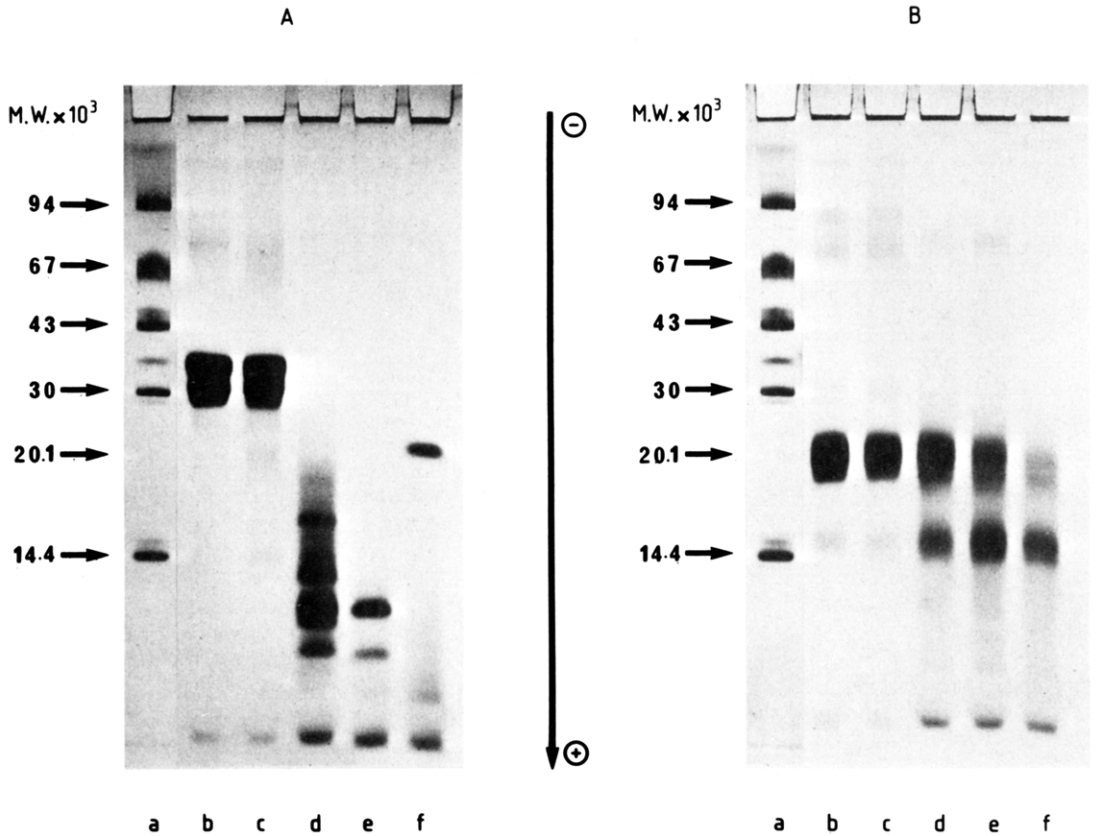

Figure 1. SDS-PAGE of proteins and peptides. See Methods for complete details. A: a, reference proteins (from top to bottom) are phosphorylase *b* (92 000/subunit), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), Kunitz soybean trypsin inhibitor (21 000), and α-lactalbumin (14 400); b, unmodified casein; c, control casein; d, sample Cn1; e, sample Cn2; and f, sample Cn3. B: a, reference proteins (see A); b, unmodified WPC; c, control WPC; d, sample WP1; e, sample WP2; and f, sample WP3.

aliquots were cooled to room temperature and stirred, turbidity was again measured as above (EAI, 80 °C). The emulsion stability was calculated by

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

where EAI(max) is the maximum value obtained either at $t = 0$ or 24 h. These values did not differ significantly. The smaller the value of Δ EAI %, the better the stability.

RESULTS AND DISCUSSION

Limited Proteolysis of Casein and Whey Proteins with Trypsin. *Casein.* Hydrolysates at three times were selected to determine solubility and emulsifying properties of the peptide mixtures: a 4.3% DH (Cn1), an 8.0% DH (Cn2), and a 9.9% DH (Cn3) obtained after 5-, 17-, and 110-min hydrolysis with E/S ratios of 0.5%, 1.0%, and 1.0%, respectively.

Whey Proteins. Three hydrolysates were selected to determine solubility and emulsifying properties of the peptide mixtures: a 2.5% DH (WP1), a 3.9% DH (WP2), and a 5.3% DH (WP3) obtained after 30-, 30-, and 18-min hydrolysis with E/S ratios of 0.50%, 0.85%, and 1.00%, respectively.

Results of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE results of unmodified, control, and enzyme-treated proteins in the presence of SDS, urea, and 2-mercaptoethanol are shown in Figures 1 and 2. Separation is based primarily on size. A plot of log (molecular weight) vs mobility of the reference proteins (Figures 1Aa,Ba, and 2Aa,Ba) provided some information on the size of peptides formed after hydrolysis.

In the absence of enzyme treatment (Figure 1Ab,c), casein separated into two major bands (MW 35 000–39 000, 30 000–34 000). Some aggregates (MW ~75 000) and several minor bands (MW ~21 000, ~25 000) were observed. After 5-min enzyme treatment (Cn1), at least 11 bands were visible (Figure 1Ad) with apparent molecular weights ranging from ~20 000 to <14 400 (~4000); two main bands at MW <14 400 (~9000) had a major intensity of dye staining. After 17-min enzyme treatment (Cn2), all the bands with an apparent molecular weight ranging from ~20 000 to <14 400 (~10 000) disappeared; the two main bands at MW <14 400 (~9000) had a lower intensity. After 110-min enzyme treatment (Cn3) it was very difficult to observe well-defined bands of low molecular weight. An unexpected band of MW ~21 000 appeared; this band was probably a contaminant since by size-exclusion chromatography no peak with this apparent molecular weight could be detected.

SDS-PAGE performed with lower molecular weight markers (Figure 2A) provided further information on the size of peptides with a molecular weight below 14 400. In these conditions the 11 bands observed for Cn1 had molecular weights ranging from 12 000 to 2500.

In summary, after trypsin treatment of casein the main bands have a high mobility and correspond to material with a molecular weight below 10 000–15 000.

In the absence of enzyme treatment (Figure 1Bb,c), whey proteins separated into two major bands (MW 18 000–21 000, 14 000) corresponding to β-lactoglobulin and α-lactalbumin, respectively. Some aggregates were observed with molecular weights ranging from 30 000–32 000, 38 000–42 000, and 67 000–78 000. After enzyme treatment, the band with apparent MW 18 000–21 000 had a de-

Table I. Emulsifying Capacity (Oil Phase Volume, %)[a] of Caseins and Whey Proteins as a Function of pH[b]

pH	control casein	Cn1	Cn2	Cn3	control WPC	WP1	WP2	WP3
1	38 ● 0.24	43 ± 0.13	43 ± 0.26	41 ± 0.14	40 ● 0.58	41 ± 0.33	39 ± 1.58	38 ± 0.32
2	46 ± 0.98	45 ± 0.96	47 ± 0.12	41 ± 1.40	41 ± 0.40	42 ± 0.27	42 ± 0.27	42 ± 1.36
3	43 ± 1.08	42 ± 0.14	42 ● 1.10	36 ± 0.34	40 ± 0.35	41 ± 0.15	41 ± 0.28	39 ± 0.77
4	29 ● 2.07	37 ± 0.34	38 ± 0.65	35 ● 1.07	37 ± 0.48	38 ± 0.18	35 ● 0.51	37 ± 1.32
5	34 ● 0.90	33 ● 0.19	38 ± 0.80	31 ± 1.30	39 ● 0.42	41 ± 0.11	41 ● 0.30	40 ± 0.30
6	43 ± 1.36	42 ● 0.28	41 ± 1.58	34 ± 0.36	41 ● 0.54	43 ± 0.10	43 ● 0.41	41 ± 0.29
7	44 ± 2.60	44 ● 0.53	43 ● 1.50	38 ± 1.60	41 ● 0.52	43 ± 0.24	43 ± 0.27	43 ● 1.50
8	47 ± 2.37	45 ● 0.38	44 ● 1.54	43 ± 0.27	41 ● 0.13	44 ± 0.34	44 ± 0.13	43 ● 1.50
9	48 ± 1.56	45 ± 0.43	45 ● 1.25	47 ± 1.06	41 ● 0.14	44 ± 0.44	45 ● 0.11	43 ● 1.50
10	53 ± 0.09	47 ● 0.80	47 ● 0.70	50 ± 0.41	41 ● 0.11	46 ● 0.52	47 ± 0.46	46 ● 0.80
11	63 ± 2.38	49 ± 0.32	52 ± 0.78	56 ● 0.47	50 ± 0.43	55 ● 0.24	73 ± 0.18	52 ± 0.47

[a] According to Acton and Saffle (1972). [b] All the data are mean values of triplicates.

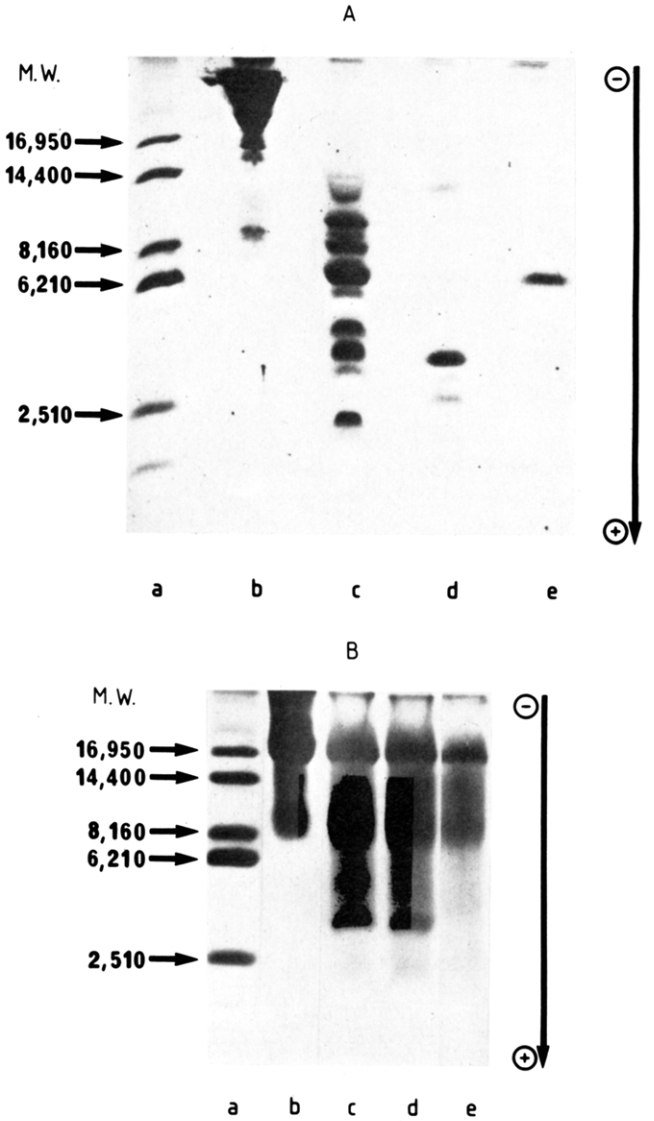

Figure 2. SDS-PAGE of proteins and peptides with lower molecular weight markers. A: a, reference proteins (top to bottom) are five polypeptides derived from the cleavage of the polypeptide chain of horse heart myoglobin (apomyoglobin), myoglobin (polypeptide backbone, 16950); myoglobin (fragment I + II, 14400); myoglobin (fragment I, 8160); myoglobin (fragment II, 6210); and myoglobin (fragment III, 2510). b–e, c–f in Figure 1A. B: a, reference proteins (see A); b–e, c–f in Figure 1B.

creasing intensity of dye staining when the DH was increasing whereas a band with apparent MW 12000–13000 had an increasing intensity of dye staining when the DH was increasing. Moreover, two bands appeared with apparent MW <14400. SDS-PAGE performed with lower molecular weight markers (Figure 2B) enabled us to further determine the apparent molecular weights of the bands below 14400 (~5600, ~3600).

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).

After hydrolysis of casein with trypsin, all the resulting peptides had molecular weights below 15000, including 30%, 44%, and 33% between 5000 and 10000 and 18%, 35%, and 66% below 5000 for Cn1, Cn2, and Cn3, respectively.

After a 2.5% DH (WP1), all the material had MW <20000 with major peaks near 18000, 13000, 9000, 5000, and 1500. After a 3.9% DH (WP2), all the material had MW <15000 with major peaks near 9000 and 5000; an increased peak was observed near 1500. After a 5.3% DH (WP3), all the material had MW <10000, with major peaks between 6000 and 2000; an always increased peak was observed near 1500.

Solubility. After partial hydrolysis (4.3%, 8.0%, 9.9%) of casein with trypsin, the peptide solution had a minimum solubility at pH 3.5–5.5, in contrast to pH 4.0–5.0 for the control casein (Figure 4). Solubility in the p*I* (isoelectric point) range increased from 0 to 45, 65, and 70% for control casein, Cn1, Cn2, and Cn3, respectively. After partial hydrolysis (2.5%, 3.9%, 5.3%) of whey proteins with trypsin, the peptide solution had a minimum solubility at pH 3.5–5.0, as control WPC (Figure 5). Solubility in the p*I* range increased from 35 to 45, 52, and 59% for control WPC, WP1, WP2, and WP3, respectively. Therefore, one may conclude that the more hydrolyzed the casein and the whey protein concentrate, the more soluble they are near the p*I*. Moreover, solubility of enzyme-treated whey proteins increased at all pHs as compared to control WPC.

Emulsifying Capacity. Emulsifying capacities of control casein solutions were measured over a range of pH values. The emulsifying capacity (EC) increased at pHs above and below 4.0–5.0 (Figure 6). The increase in EC was much more pronounced at values above pH 4.5 as compared to that below pH 4.5. The minimum at pH 4.5 is presumably because of flocculation due to minimum protein solubility. This pH corresponds to the isoelectric point of the caseins. A very high value was obtained at pH 11.0; 220 g of oil was necessary to reach the point of oil inversion in the emulsion, characterized by an infinite resistance. Such a high value has already been reported (Pearson et al., 1965).

After trypsin treatment (Figure 6; Table I), an improvement of EC was observed only at pH 4.0–5.0. For the pH values above the p*I*, the EC of enzyme-treated casein was less than that observed for control casein. In the range pH 5.5–8.0, the more hydrolyzed the casein, the less the emulsifying capacity.

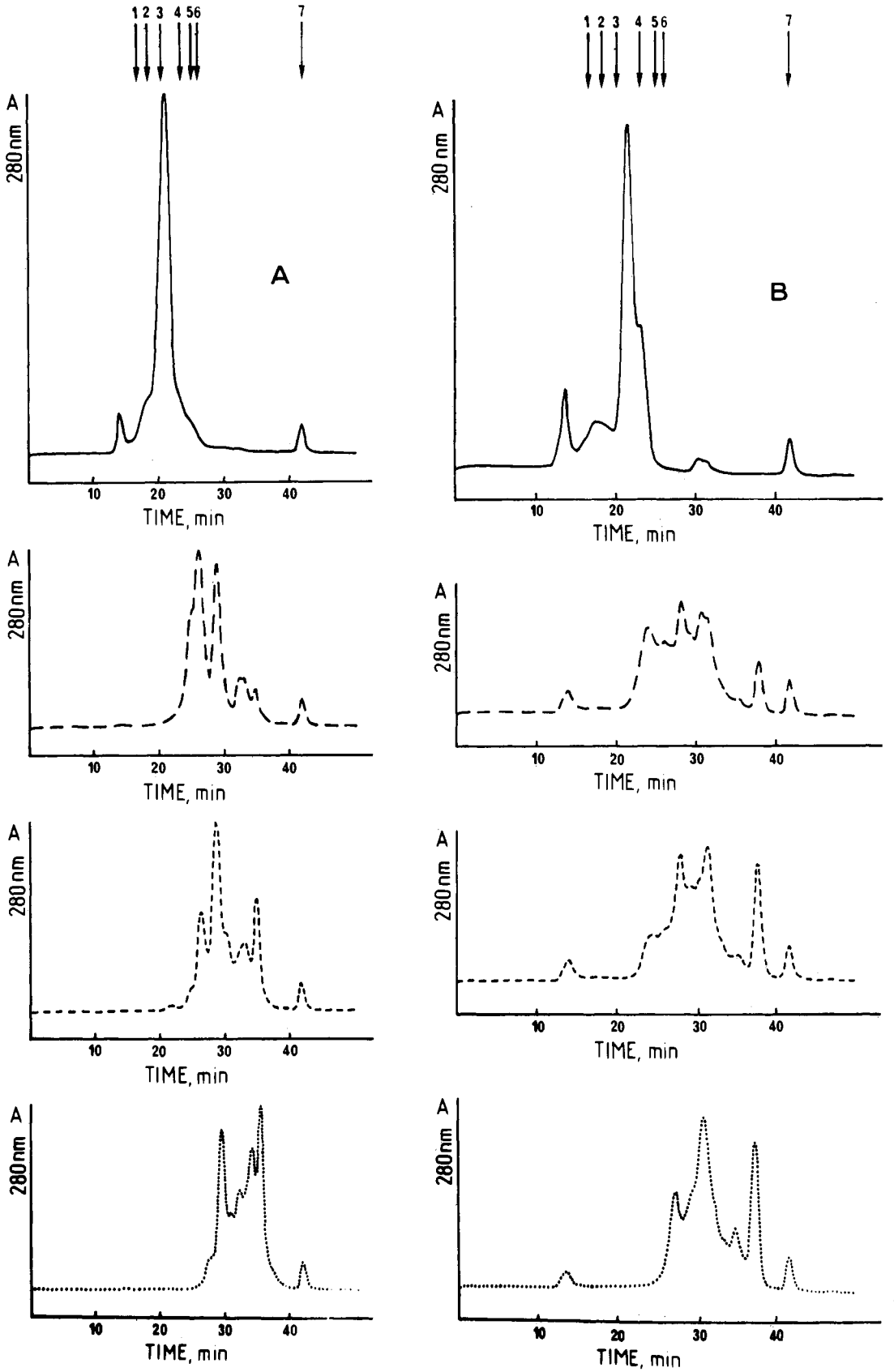

Figure 3. Size-exclusion chromatography in a Superose 12 FPLC Pharmacia column (1.5 × 30 cm). Chromatography was performed in 50 mM phosphate buffer, pH 7.0, containing 6 M urea and 0.15 M NaCl. The arrows indicate elution time of standard proteins and 2-mercaptoethanol: 1, bovine serum albumin, MW 67 000; 2, ovalbumin, MW 43 000; 3, carbonic anhydrase, MW 30 000; 4, soybean trypsin inhibitor, MW 20 100; 5, α-lactalbumin, MW 14 400; 6, cytochrome *c*, MW 12 400; 7, 2-mercaptoethanol, MW 78. A. Casein and casein derivatives. Symbols used: —, control casein; – –, sample Cn1; - - -, sample Cn2; ···, sample Cn3. B. Whey proteins and whey proteins derivatives. Symbols used: —, control WPC; – –, sample WP1; - - -, sample WP2; ···, sample WP3.

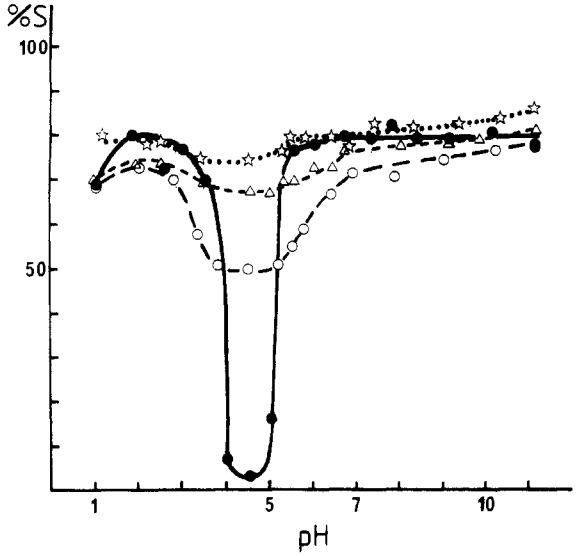

Figure 4. Solubility of control and protease-modified caseins as a function of pH. Solubility was measured after a 30-min equilibration period at room temperature (20 °C). The solubility is expressed as percent total protein (0.1%) in solution. Symbols used: ●—●, control casein; ○—○, sample Cn1; △--△, sample Cn2; ☆···☆, sample Cn3.

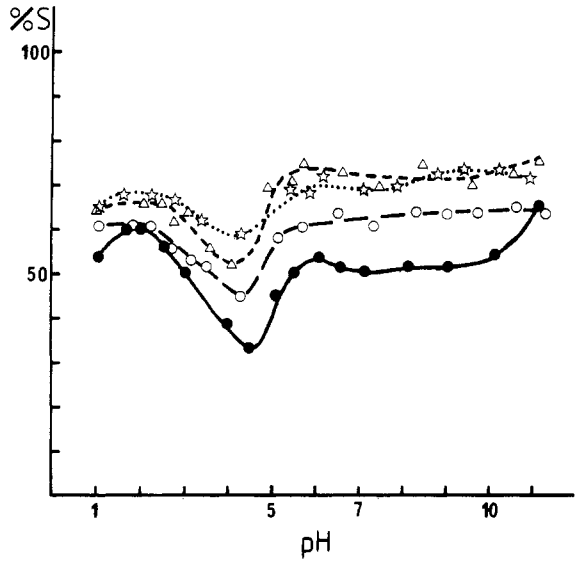

Figure 5. Solubility of control and protease-modified whey proteins as a function of pH (see Figure 4 for conditions). Symbols used: ●—●, control whey proteins; ○—○, sample WP1; △--△, sample WP2; ☆···☆, sample WP3.

Different results were obtained with whey proteins. In spite of a small decrease near the p*I*, EC of control WPC was almost constant in the range pH 1.0–9.0 (80–90 g of oil added). By contrast, at alkaline pH, an increase of EC was observed for control WPC (120 g of oil was necessary to reach the point of oil inversion in the emulsion) (Figure 7).

After trypsin treatment (Figure 7; Table I), an improvement of EC was especially observed at alkaline pH as compared to control WPC (360, 145, and 130 g of oil added to reach the point of oil inversion in the emulsion, for WP2, WP1, and WP3, respectively).

Although a good correlation has often been observed between the emulsifying capacity and hydrophobicity of proteins, it should be pointed out that the emulsifying ability of proteins could also depend on the distribution of hydrophobic and hydrophilic residues on the polypeptide chain. At pH 11, a conformational change of the polypeptide chain (unfolding) occurred, demasking some hydrophobic residues, and consequently those residues should interact with lipids. These factors may be responsible for the very high emulsifying capacity of WP2 at alkaline pH.

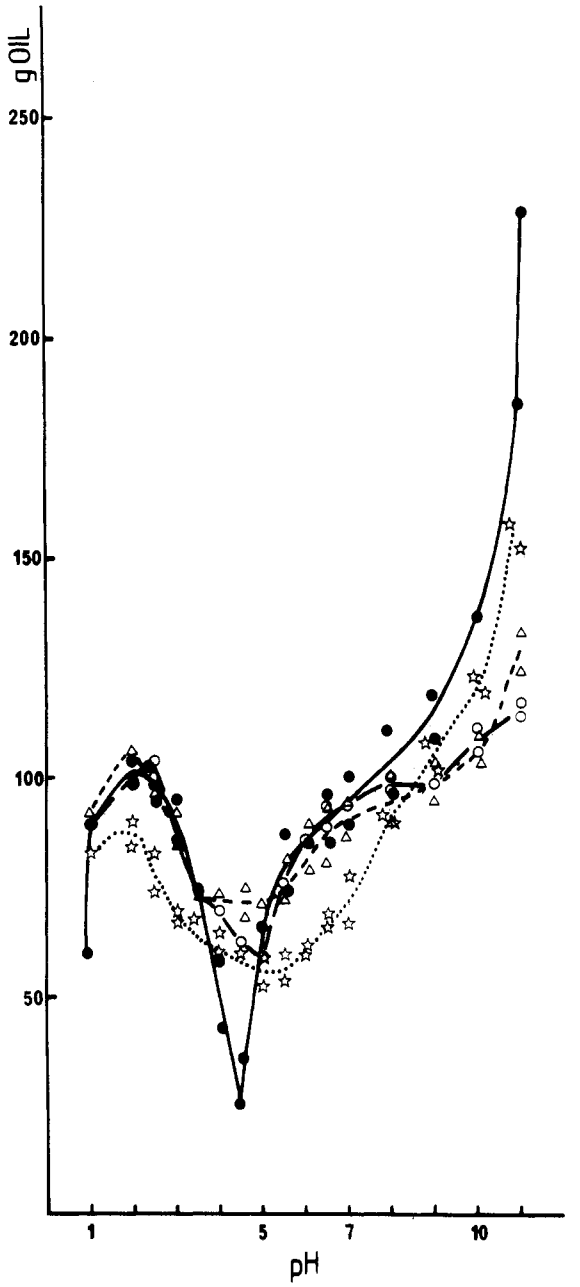

Figure 6. pH–emulsifying capacity curves of control and protease-modified caseins. Results are expressed as grams of emulsified oil/120 mg of protein in water. Symbols as in Figure 4.

Emulsifying Activity. Emulsions were prepared from control casein solutions over a range of pH values. The emulsifying activity index increased above and below pH 4.0–5.0 (Figure 8). The increase in EAI was much more pronounced at values higher than pH 5.5, as compared to that below 4.0.

After trypsin treatment, no reproducible emulsions were obtained in the range pH 3.5–5.5, 3.2–5.5, and 2.5–5.5 for Cn1, Cn2, and Cn3, respectively. The base-line absorbance is not 0, since the oil/protein/water system had a small absorbance in relation to the blank. Most of the absorbance values below 0.05–0.10 were not significant. At pH 2.0, an increase of EAI was observed as compared to the

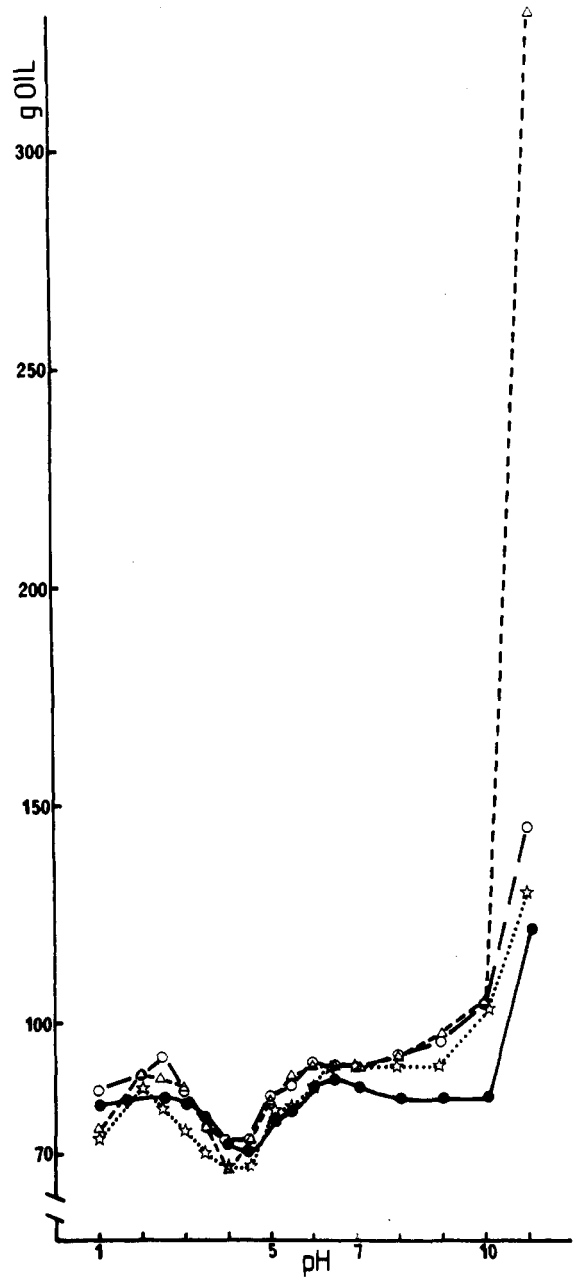

Figure 7. pH–emulsifying capacity curves of control and protease-modified whey proteins (see Figure 6 for conditions). Symbols as in Figure 5.

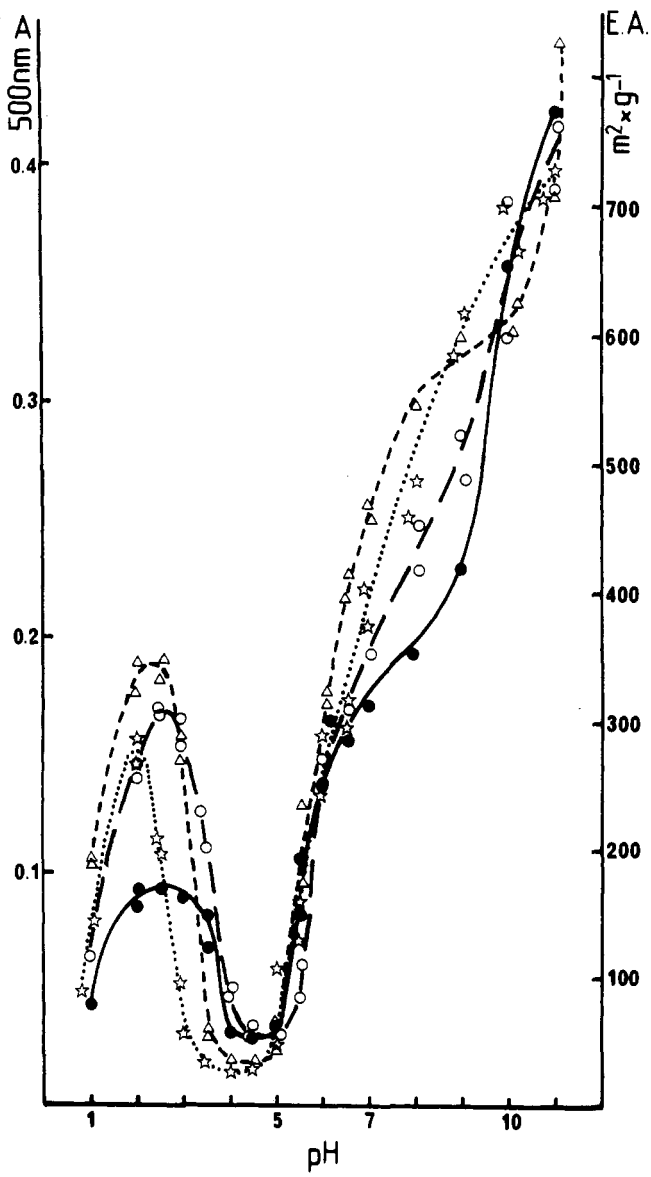

Figure 8. Absorbance at 500 nm and emulsifying activity index of control and protease-modified caseins as a function of pH. See Methods for conditions. Symbols as in Figure 4.

control casein, the maximum value being obtained with Cn2. From pH 6.0 to 9.5–10.0, the EAI of the hydrolysates was somewhat larger than the casein control, maximum improvement being observed with Cn2.

For control WPC, all the emulsions broke in the range pH 1.0–10.0. Although the EAI increased above and below pH 4.0–5.0 (Figure 9), the increase in EAI was more pronounced at values greater than pH 5.5, as compared to that below pH 4.0. However, as compared to control casein, EAI of WPC were much lower. A large increase of EAI was observed at pH 11.

After trypsin treatment, all the emulsions broke in the range pH 3.5–5.0. In the ranges pH 1.0–3.5 and 5.0–11.0, the EAI showed a large increase as compared to control whey proteins. As for trypsin-treated casein, EAI increased when DH increased until an optimum and then decreased (EAI(WP2) > EAI(WP1) > EAI(WP3)). After trypsin treatment, the pH–EAI curves of whey proteins look much like those obtained with casein.

Emulsifying Stability. After 24-h storage and heating of the emulsion, a large pH-dependent decrease of emulsifying activity index (30–75%) was observed for values above pH 5.5, for samples Cn1, Cn2, and Cn3. Under the same conditions, EAI of control casein showed a 2–7% decrease (Table II).

All the emulsions of control whey proteins broke, except above pH 10.0, and EAI of trypsin-treated whey proteins showed a pH-dependent decrease (0–36%) for values above pH 5.5 (Table II). Generally, whatever the decrease of EAI after thermal processing of the emulsion (80 °C for 30 min), the emulsifying activity of protease-treated whey proteins was higher as compared to EAI of control WPC.

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 and whey proteins with trypsin, solubility was increased particularly in the p*I* range, with 0%, 45%, 65%, and 70% solubilities for control casein, Cn1, Cn2, and Cn3, respectively, and with 35%, 45%, 52%, and 59%

Table II. Emulsion Stability of Caseins and Whey Proteins after 24 h at 20 °C and 30 min at 80 °C[a]

pH	control casein	Cn1	Cn2	Cn3	control WPC	WP1	WP2	WP3
1.0	–	–	–	–	–	–	–	–
2.0	50 ± 0.13	7 ± 0.28	12 ± 0.25	35 ± 0.20	–	20 ± 0.50	44 ± 0.16	62 ± 0.11
3.0	12 ± 0.18	9 ± 0.37	36 ± 0.26	61 ± 0.50	–	4 ± 0.20	3 ± 0.19	25 ± 0.18
4.0	–	–	–	–	–	–	–	–
4.5	–	–	–	–	–	–	–	–
5.0	–	–	–	–	–	15 ± 1.40	0	5 ± 0.18
5.5	–	–	–	–	–	6 ± 0.10	0	7 ± 0.13
6.0	6 ± 0.36	65 ± 0.19	75 ± 0.27	69 ± 0.23	–	3 ± 0.30	11 ± 0.55	5 ± 0.15
7.0	7 ± 0.20	52 ± 0.05	66 ± 0.33	47 ± 0.20	–	4 ± 0.01	10 ± 0.24	4 ± 0.09
8.0	4 ± 0.11	55 ± 0.02	57 ± 0.18	30 ± 0.81	–	5 ± 0.54	14 ± 0.38	16 ± 0.54
9.0	5 ± 0.21	53 ± 0.11	53 ± 0.32	39 ± 0.04	–	4 ± 0.10	11 ± 0.33	26 ± 0.11
10.0	5 ± 0.21	56 ± 0.08	48 ± 0.09	33 ± 0.31	3 ± 0.10	7 ± 0.10	15 ± 0.05	22 ± 0.04
11.0	2 ± 0.04	43 ± 0.25	35 ± 0.28	37 ± 0.19	4 ± 0.40	36 ± 0.20	22 ± 0.54	26 ± 0.33

[a] Results are expressed as percent difference between EAI(max) and that after thermal processing (see Methods). –, not determined because of emulsion collapse.

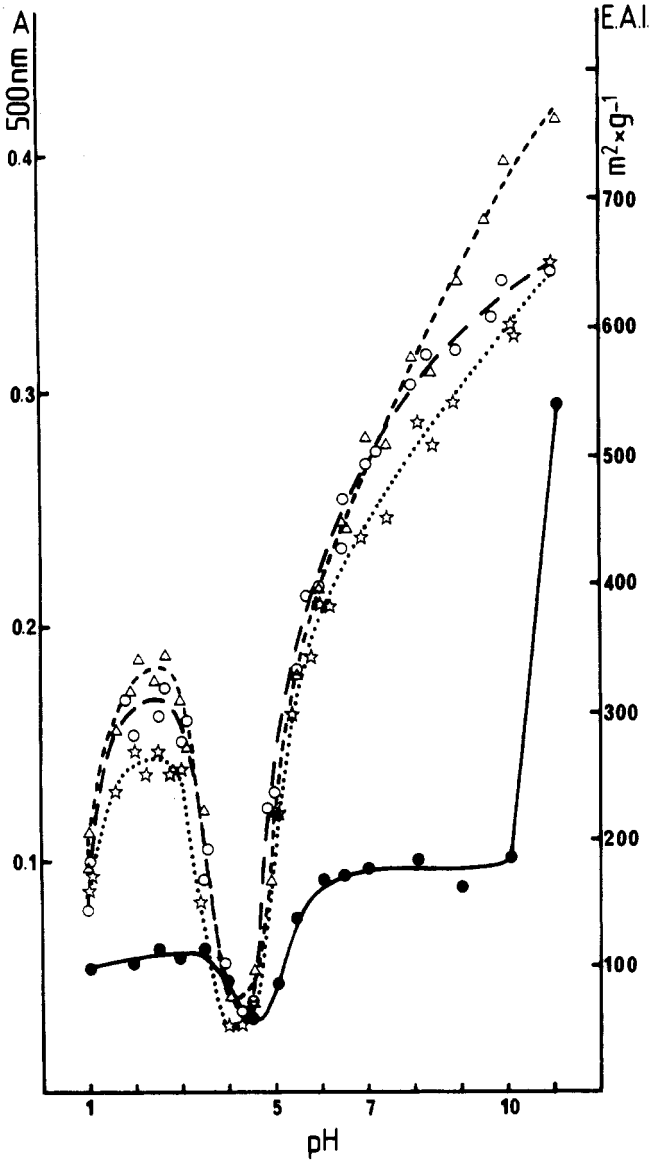

Figure 9. Absorbance at 500 nm and emulsifying activity index of control and protease-modified whey proteins as a function of pH. Symbols as in Figure 5.

solubilities for control whey proteins, WP1, WP2, and WP3, respectively. In the pH range around p*I*, it was not possible to obtain a reproducible emulsion. These results near the p*I* show that increasing the solubility did not increase the emulsifying activity. Similar results were obtained with casein treated with *S. aureus* V8 protease (Chobert et al., 1988).

The shape of the pH–emulsifying capacity curves of control casein and whey proteins was not quite comparable to that of solubility, particularly in alkaline conditions where we observed a large increase of EC whereas solubility was stable.

After trypsin treatment, although the emulsifying capacity of casein derivatives increased in the p*I* range, the more hydrolyzed sample (Cn3), which was the most soluble, gave a decreased EC, as compared to other hydrolyzed samples (Cn1, Cn2). Trypsin-treated whey proteins WP3, the most hydrolyzed sample with the highest solubility in the p*I* range, had a lower emulsifying capacity as compared to other hydrolyzed samples (WP1, WP2) and control whey proteins.

A positive correlation between solubility and emulsifying capacity of proteins has been reported (Crenwelge et al., 1974; Volkert and Klein, 1979). It is nevertheless worth stressing that, in a number of other examples, a poor correlation was observed (Wang and Kinsella, 1976; McWatters and Cherry, 1977; McWatters and Holmes, 1979; Aoki et al., 1981).

Solubility of casein was improved in the p*I* range after partial hydrolysis. However, no emulsion activity was obtained in this pH range. For pH values below and above p*I*, whereas solubility was not changed for casein derivatives, an improvement of emulsifying activity was observed, with an optimum after limited hydrolysis (sample Cn2). However, the emulsifying stability of the casein derivatives was lower than that of control casein. There is the possibility that the majority of the peptides are not amphiphilic enough as is needed for good emulsifying stability. In a preceding paper (Chobert et al., 1988) we had shown that, after partial hydrolysis (2.0 and 6.7% DH) of casein with *S. aureus* V8 protease, emulsifying activity and stability of casein derivatives decreased as compared to control casein. In both cases (enzymic modification with *S. aureus* V8 protease or trypsin), the size of the resulting peptides seems to be in the same range, according to the results obtained by SDS-PAGE and by size-exclusion chromatography. Therefore, the specificity of the protease is quite important. Such differences were also observed by Adler-Nissen and Olsen (1979) with soy proteins; they 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 limited proteolysis increased the whipping expansion (12-fold at 3% hydrolysis) 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.

Whey proteins are known to have a globular conformation stabilized by polymerization. Trypsin treatment

results in a breakdown of this conformation, and consequently hydrophobic residues buried in the core of the molecule are now able to interact with lipids during the formation of an emulsion, as is the case for control casein. This phenomenon may explain the larger improvement of emulsifying activity observed after partial hydrolysis as compared to the little variation observed with casein hydrolysates. A limited DH was necessary to largely improve emulsifying activity, since extensive hydrolysis may lead to nonamphiphilic peptides. In order to maintain good emulsifying properties, the apparent molecular weight of peptides should not be lower than ~5000.

Whey protein derivatives were more stable than casein derivatives and always gave a higher emulsifying activity as compared to control whey proteins. Although functional properties could be improved by controlled limited proteolysis of proteins, further work is needed in order to predict the relationships between the structure of a protein and its functional properties. Separation and characterization of the peptides will be required. However, Shimizu et al. (1986) demonstrated that a peptide of 23 residues, purified from the peptic hydrolysate of α_{s1}-casein, also had very complex emulsifying properties, being affected by coexistent peptides, emulsification conditions, and other factors. Recently, Lee et al. (1987) isolated two peptides, one hydrophilic and the other hydrophobic, from the hydrolysate (trypsin, chymosin) of β-casein. The emulsifying activities of both peptides depended on the pH range. In summary, more knowledge of peptide functionality is essential.

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Soybean Metal-Binding Proteins: Isolation of a Phosphatase That Inhibits Calmodulin Activity

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Soybean seeds and seedlings contain monophosphatase (MP), phosphodiesterase (PDE), and a calmodulin (CAM) that stimulates PDE activity. Interactions of these proteins likely regulate phosphate metabolism in the seed. This paper identifies proteins that interact with CAM and defines the function of CAM in soybeans. We find that an MP enzyme can inhibit CAM activity and is associated with CAM in soybean whey. MP enzyme is separated into a single peak by DEAE chromatography in the presence of a calcium chelator, EGTA, and further chromatography on a calmodulin affinity column. Its binding to the calmodulin affinity column is reversible and depends on the presence of Ca^{2+} ions. HPLC, gel electrophoresis, and gel filtration analyses of the MP enzyme show that it contains two subunits (24 and 20 kDa), but neither of the subunits alone exhibits phosphatase activity. The 20-kDa subunit retains the ability to inhibit CAM stimulation of PDE. Recombination of the two subunits yields MP activity that can be further enhanced by the addition of CAM. MP activity appears to be less stable than the CAM inhibitor activity.

As part of studies on CAM in soybeans, we isolated the protein from soybean whey by chromatographing extracts on DEAE and then subjecting collected fractions to affinity chromatography on W-7 agarose to obtain pure CAM. The purified protein is similar to calmodulin from spinach and bovine brain in terms of ability to stimulate PDE activity and electrophoretic mobility as a 18-kDa protein (unpublished result). Its behavior during purification suggests that it is associated with other calcium-binding proteins in the whey, particularly in the presence of Ca^{2+} ions. A calcium chelator (EGTA) is required during DEAE chromatography to separate CAM from other whey proteins. We now have evidence that the proteins to which CAM binds include an MP enzyme that hydrolyzes *p*-nitrophenyl phosphate. This MP enzyme also inhibits the stimulatory action of CAM on PDE. It is a multisubunit

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