Emulsifying Properties of Peptides Obtained from the Hydrolyzates of β -Casein

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A hydrophobic peptide of 17 residues, β -CN (f193~209), and a hydrophilic peptide of 25 residues, β -CN (f1~25), were isolated from enzyme hydrolyzates of bovine β -casein. The emulsifying activity (EA) of both peptides was low at a neutral pH. In the acidic or alkaline condition, however, β -CN (f193~209) showed high EA values. β -CN (f1~25) also showed high EA values at acidic pHs. These peptides are shown to be more surface active at pH 3 than at pH 7.

In the preceding papers,^{1,2)} we reported on the emulsifying properties of an N-terminal 1~23 peptide of α_{s1} -casein. This peptide, α_{s1} -CN (f1~23), showed some unique emulsifying properties: remarkable pH-dependence, concentration-dependence, and a synergistic effect with other peptides. However, more information on the emulsifying properties of many other peptides is required to understand the characteristics of the peptide as an emulsifier.

Bovine β -casein, whose primary structure has been well established,^{3,4)} has distinctive amphipathic structure in the molecule. Its N-terminal region is hydrophilic, while the C-terminal region is hydrophobic. β -casein is, therefore, considered to be a good source to prepare peptides of differing hydrophobicity.

In the present study, we isolated two peptides, one hydrophilic and the other hydrophobic, from the hydrolyzates of β -casein and investigated their properties, especially for emulsification.

MATERIALS AND METHODS

Preparation of β -casein. Crude β -casein was prepared from fresh raw skin milk according to the method of Fox and Guiney.⁵⁾ This β -casein was purified on a DEAE-

Sephacel column ($\phi 3.5 \times 20$ cm). The elution of β -casein from the column was carried out with 0.025 M imidazole-HCl buffer (pH 6.5) containing 4 M urea under a linear gradient of NaCl (0~0.4 M).

Preparation of peptides from β -casein.

i) β -CN (f193~209). A 2% solution of β -casein in 0.01 M imidazole-HCl buffer (pH 6.5) was incubated with chymosin (Difco) at 10°C for 3 hr (E/S ratio, 1:50, w/w) and then heated for 5 min at 80°C to inactivate the enzyme. The solution was acidified to pH 4.6 with 0.1 N HCl and then centrifuged at $1,840 \times g$ for 10 min. The supernatant was adjusted to pH 6.5 and applied to a DEAE-Sephacel column ($\phi 2.6 \times 20$ cm) equilibrated with 0.01 M imidazole-HCl buffer (pH 6.5). β -CN (f193~209) was eluted by the buffer without retardation. The peptide fraction was repeatedly diluted and concentrated on a G-01T membrane filter (ULVAC Service Corp.) at 4°C until free from salts. The solution was then freeze-dried.

ii) β -CN (f1~25). The β -CN (f1~25) peptide was isolated according to the procedure of Peterson *et al.*⁶⁾ with slight modification. A 2% solution of β -casein in 0.1 M ammonia-formic buffer (pH 8.05) was incubated with trypsin (trypsin-TPCK, Worthington) at 20°C for 10 hr (E/S ratio, 1:5000, w/w). The solution adjusted to pH 4.7 was centrifuged at $1,840 \times g$ for 10 min. The clear supernatant was then made 0.25% with respect to BaCl_2 by the addition of a 10% BaCl_2 stock solution, before an equal volume of absolute ethanol was added. The crude barium phosphopeptide was precipitated by centrifugation and dried with acetone and ether. It was further purified by adjusting the pH to 3.5 $\rightarrow$ 2.0 $\rightarrow$ 3.5. The precipitate at pH 3.5 was resolubilized in 0.025 M imidazole-HCl buffer (pH 6.5) containing ethylenediaminetetraacetic acid (EDTA).

The solution was adjusted to pH 6.5 and fractionated on a DEAE-Sephacel column ($\phi 2.1 \times 18$ cm) with 0.025 M imidazole-HCl buffer (pH 6.5) under a linear gradient of NaCl (0~0.6 M). The peptide fraction was desalted by passage through Sephadex G-15 (Pharmacia Fine Chemicals Co.) and then freeze-dried.

High-performance liquid chromatography (HPLC). A high-performance liquid chromatograph (Jasco Trirotor SR-2) was fitted with a Lichrosorb RP-18 column ($\phi 4.0 \times 250$ mm, Merck) and a UV detector at a wavelength of 230 nm. The sample was eluted with 0.1% trifluoroacetic acid (TFA)-acetonitrile as a mobile phase at a flow rate of 1 ml/min. The concentration of the mobile phase modifier (acetonitrile) was increased linearly from 0~95% over 60 min (1.58%/min).

Amino acid analysis. A peptide separated on HPLC was evaporated to remove the TFA and acetonitrile, and was then dissolved in 6 N HCl. The solution was degassed and hydrolyzed at 110°C for 24 hr. The hydrolyzate was analyzed with an automatic amino acid analyzer (Hitachi Model 835).

Emulsifying activity. Emulsifying activity was evaluated according to the procedure described by Pearce and Kinsella⁷⁾ with slight modification. An emulsion was prepared by homogenizing a 2% (w/w) peptide solution and 20% (w/w) soybean oil (Sanko Pharmaceutical Co., Japan) at 30°C with a Polytron PTA-7 (Kinematica, Switzerland) for 3 min at full speed (19,500 rpm). The emulsion was immediately diluted with 0.1% SDS and its turbidity was measured at 500 nm. Emulsifying activity is presented in terms of the emulsifying activity index (EAI).

Solubility. The solubilities of peptides were measured at pH 3~10. Peptide solutions of 2% concentration (w/v) in 0.1 M NaCl were centrifuged at $8,000 \times g$ for 30 min. The peptide concentration of the supernatant was determined by the procedure of Lowry *et al.*, the solubility being expressed as (peptide concentration of the supernatant/peptide concentration of the initial solution) $\times 100$ (%).

Surface tension. The surface tension of the peptide (protein) solutions was monitored with a Wilhelmy plate-type surface tensiometer (Kyowa CBVP type A-3) at 20°C. The pH of each peptide solution (0.05%, w/v) was adjusted to 3 or 7.

Electron microscopic observation. The electron microscopic observation was performed using a scanning electron microscope (SEM, HITACHI BH-2 RFK) equipped with a cryogenic system. An emulsion was mixed with two volumes of a 2% agar solution at 45~50°C and allowed to stand for 15~30 min. The gel was sliced to a 1~2 mm thickness and soaked in 50% ethanol for 15 min. The

ethanol-treated gel slice was set in the sample chamber of the microscope and frozen by liquid nitrogen for 20 sec. The sample was then cleaved by a cold knife edge and allowed to etch for an appropriate time. The surface of the sample was finally coated with platinum-palladium and examined with a Hitachi S-800 scanning electron microscope.

RESULTS

Isolation of the peptides

The β -CN (f193~209) fraction was obtained from the chymosin hydrolyzate of β -casein. The yield was about 40 mg/g of β -casein. The peptide fraction showed no band on slab-polyacrylamide gel electrophoresis (PAGE, data not shown), but produced one peak by HPLC analysis on an ODS-column as shown in Fig. 1. The eluate corresponding to the peak was collected and its amino acid composition was analyzed. The amino acid composition of this fraction (Table I) indicates that this peptide corresponded to the C-terminal 193~209 residues of β -casein.

The β -CN (f1~25) fraction was obtained from the trypsin hydrolyzate of β -casein. The peptide fraction showed no band on slab-PAGE (data not shown). The HPLC pattern of this fraction on an ODS-column is shown in Fig. 2. Only one peak was observed, indicating the high purity of this fraction. The amino acid analysis of the eluate corresponding to the

FIG. 1. HPLC of the β -CN (f193~209) Peptide Fraction on a Lichrosorb RP-18 Column ($\phi 4.0 \times 250$ mm).

Primary solvent, 0.1% trifluoroacetic acid; secondary solvent, acetonitrile. The gradient profile is shown by the dotted line.

TABLE I. AMINO ACID COMPOSITION OF THE PEPTIDES OBTAINED FROM HYDROLYZATES OF β -CASEIN

Amino acid	β -CN (f193~209)		β -CN (f1~25)	
	Molar ratio	Nearest ^a integer	Molar ratio	Nearest ^a integer
Asx	0.20	—	1.21	1 (1)
Thr	0.10	—	1.03	1 (1)
Ser	0.26	—	4.02	4 (5)
Glx	2.49	2 (2)	7.01	7 (7)
Pro	4.42	4 (4)	1.22	1 (1)
Gly	2.24	2 (2)	1.16	1 (1)
Ala	0.07	—	—	—
Cys	0.11	—	—	—
Val	3.23	3 (3)	1.92	2 (2)
Met	—	—	—	—
Ile	1.59	2 (2)	1.78	2 (2)
Leu	1.15	1 (1)	3.23	3 (3)
Tyr	1.00	1 (1)	—	—
Phe	0.98	1 (1)	—	—
Lys	0.20	—	—	—
His	0.06	—	—	—
Arg	1.00	1 (1)	2.12	2 (2)
Total residue	17 (17)		24 (25)	

^a Theoretical values are shown in parentheses.

FIG. 2. HPLC of the β -CN (f1~25) Peptide Fraction on a LiChrosorb RP-18 Column.

The conditions are the same as in Fig. 1.

peak (Table I) indicates that this fraction corresponded to the N-terminal 1~25 residues of β -casein. The low values of Ser are considered to be due to its partial degradation during HCl hydrolysis as described by Peterson *et al.*⁶⁾ The yield of β -CN (f1~25) was about 70 mg/g of β -casein.

TABLE II. EMULSIFYING ACTIVITY AND STRUCTURAL PROPERTIES OF THE PEPTIDES

	EAI (m ² /g)	Average hydrophobicity ^a	Molecular weight
β -CN (f193~209)	8.2 ± 0.8	1,768	1,880
β -CN (f1~25)	10.4 ± 0.6	938	3,115
α_{s1} -casein	21.1 ± 0.2	1,170	23,614

^a Average hydrophobicity (HI) was calculated according to the method of Bigelow.⁹⁾

FIG. 3. Effect of pH on the Emulsifying Activity and Solubility of the Peptides.

a) β -CN (f193~209); b) β -CN (f1~25). Emulsifying activity is expressed as the emulsifying activity index (EAI).

TABLE III. SURFACE TENSION^b OF THE PEPTIDES

	β -CN (f193~209) ^a	β -CN (f1~25) ^a
pH 7	54.4	56.1
pH 3	50.1	51.8

^a Peptide concentration, 0.05% (w/v).

^b Surface tension (equilibrium).

Emulsifying properties of the peptides

The emulsifying activity index (EAI) of the two peptides was determined by comparing with that of α_{s1} -casein, and are shown in Table II. The average hydrophobicity of the peptides calculated by the procedure of Bigelow⁹⁾ is also shown in Table II. The β -CN (f1~25) peptide was hydrophilic, whereas the β -CN (f193~209) peptide was very hydrophobic.

The EAI of β -CN (f1~25) was low when a 2% peptide solution (at pH 7) was used for measurement. The hydrophobic peptide, β -CN

(f193~209) also showed a remarkably low EAI.

Effect of pH on the emulsifying activity

The emulsifying activity and solubility of the two peptides at various pH values are shown in Fig. 3. Both peptides were soluble over the range of pH from 3 to 10.

The EAI of β -CN (f193~209) was very low at pH 5.5~7, but it showed high EAI values at

less than pH 4 and more than pH 8. On the other hand, β -CN (f1~25) showed a high value only at less than pH 4.

Surface tension

The surface tension of the two peptide solutions after reaching equilibrium (3~5 hr after the initial measurement) was measured at pH 7 and 3. The peptides seemed to be slightly more surface active at pH 3 than at pH 7

FIG. 4. Phase Contrast Microscopic Observations of Emulsions Prepared with the Peptides.

a) β -CN (f193~209), pH 7; b) β -CN (f193~209), pH 3; c) β -CN (f1~25), pH 7; d) β -CN (f1~25), pH 3.

FIG. 5. Scanning Electron Micrographs of Emulsions Prepared with the Peptides.

a) β -CN (f193~209), pH 3; b) β -CN (f1~25), pH 7; c) β -CN (f1~25), pH 3.

(Table III).

Microscopic observation

Emulsions prepared at pH 7 and 3 were observed by phase contrast and scanning electron microscopy. β -CN (f193~209) did not produce a stable emulsion at pH 7 (Fig. 4a), while a stable, fine dispersion of the oil droplets was observed at pH 3 (Fig. 4b), in good agreement with Fig. 3a. The phosphopeptide was observed to form a stable emulsion at pH 7, although the oil droplet size was relatively large (Fig. 4c). The droplet size of β -CN (f1~25) decreased at pH 3 (Fig. 4d) in agreement with Fig. 3b. The scanning electron microscopy demonstrated that the oil droplets in the emulsions were covered with envelopes (Fig. 5), these envelopes being considered to be adsorbing layers of the peptides. Stabilization of the oil droplets could be achieved by these peptide films.

DISCUSSION

Many reports concerning the emulsifying properties of proteins have been published. However, the mechanism of emulsification by proteins remains obscure. This is mainly because of the highly complex structure of proteins and their dynamic properties at the oil/water interface during emulsification.

Peptides have simpler structure compared with proteins. Peptides of low molecular weight are considered to have fewer tertiary structures and a less rigid conformation. The contribution of the primary structure to the emulsifying property could be more pronounced in peptides. β -CN (f193~209) is a highly hydrophobic peptide containing only one ionic amino acid residue, while β -

CN (f1~25) is highly hydrophilic, being rich in acidic amino acid residues. A marked difference in the emulsifying properties of these two peptides was therefore expected.

The present study, however, demonstrates that both peptides had very low emulsifying activity at a neutral pH. In the previous paper,²⁾ we observed that an amphipathic peptide corresponding to the N-terminal 23 residues of α_{s1} -casein also showed a low EAI, although this peptide portion plays an important role in the interaction of α_{s1} -casein with emulsified oil.¹⁰⁾ The fact that all of these peptides do not possess good emulsifying properties irrespective of the amino acid composition and hydrophobicity suggests that the chain length of the peptide seems to be a predominant factor for emulsification. Peptides of about 20 residues might be too short to have good emulsifying properties. Many authors have studied the functional properties of protein hydrolyzates,^{11~15)} and postulated that the peptide chain length in the hydrolyzates should not be too small for achieving good emulsifying properties. The low emulsifying activities of the peptides used in this study may be due to the short chain length of these peptides.

In spite of the short chain length, the peptides showed high emulsifying activity when emulsification was carried out in the acidic or alkaline condition. It is well known that the pH change often influences the solubility of proteins, thereby affecting the emulsifying properties. However, the solubility of the two peptides used in this study did not change within the range of pH 3~10, suggesting that the emulsifying properties of the peptides did not relate to the solubility.

β -CN (f193~209) showed high EAI values

a) β -CN (f193-209)

¹⁹³Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val²⁰⁹

b) β -CN (f1-25)

¹Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-Ser-Leu-Ser-Ser-Ser-Glu-Glu-Ser-Ile-Thr²⁵-Arg
P P P P

FIG. 6. Primary Structures of the Peptides.

at above pH 8 and below pH 4. This result is unaccountable because β -CN (f193~209) is a peptide having only three ionizable amino acid residues, 193Tyr (N-terminal), 202Arg and 209Val (C-terminal). Drastic changes to the structure of β -CN (f193~209) by changing pH seem to be unlikely within the pH range of 3~10. However, the low surface tension of the peptide solution at pH 3 (Table III) suggests that some structural change of the peptide occurred in the acidic pH region. β -CN (f1~25) also showed a high EAI and low surface tension at pH 3. Unlike β -CN (f193~209), the structure of β -CN (f1~25) is considered to be affected by changing pH, since it contains 7Glu, 4phospho-Ser and 2Arg residues. At pH 3, dissociation of the Glu residues must be suppressed, which makes the N-terminal portion of β -CN (f1~25) less hydrophilic. The amphipathic property of the β -CN (f1~25) molecule induced by such a change in the ionizable groups might have caused the increase in the emulsifying and surface activities. Secondary or tertiary (if any) structures of peptides and peptide-peptide association at the oil/water interface should be carefully considered as well to resolve the unaccountable properties of peptides for emulsification. In particular, peptide-peptide association must be important. The particularly thick adsorbing films observed by electron microscopy suggest that a high degree of association among the peptides might have occurred on the oil droplet

surface. A more detailed investigation of this suggestion is necessary to elucidate the mechanism for emulsification by the peptide.

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