Complexes of Casein Phosphopeptide and Calcium Phosphate Prepared from Casein Micelles by Tryptic Digestion

Tomotada ONO, Toshihiko OHOTAWA,* and Yasushi TAKAGI**

Department of Bioscience and Technology, Iwate University, Morioka 020, Japan
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Casein phosphopeptide (CPPcm), which inhibits the precipitation of calcium phosphate in the intestines, was prepared as a CPPcm–calcium phosphate complex from casein micelles (casein–calcium phosphate conjugates). CPPcm was eluted as seven peaks from a Q-sepharose FF column, the major peaks being identified and agreeing with those of the phosphopeptides (CPPcm) from acid-precipitated casein. The major components of CPPcm were $\alpha_{s1}$-CN-5P(59–79) and $\beta$-CN-4P(1–25), CPPcm containing more $\alpha_{s1}$-CN-5P (f59–79) than CPPcn. The complexes of CPPcm and calcium phosphate contained twice the quantity of calcium phosphate as those of CPPcn.

Mammalian infants, especially ruminants, grow rapidly, this growth being supported by milk, which is rich in calcium phosphate. Calcium phosphate is a major component of animal bones. Casein, which is a major component of milk proteins, makes stable complexes (casein micelles) by binding calcium phosphate beyond the limit of calcium phosphate solubility to its phosphoryl residues. An sufficient supply of calcium phosphate to infants is provided by casein micelles. The phosphorylated segments of caseins are called casein phosphopeptides. After drinking milk, the casein is digested into peptides in the intestines. Casein phosphopeptides (CPP) have been detected in the intestinal contents of pigs and rats fed on a casein diet, and also in the feces of rats. It has been suggested that CPP may inhibit the precipitation of calcium phosphate in the ileum, and increase the amount of soluble calcium available for absorption.

CPP has conventionally been prepared from casein and $\beta$-casein by tryptic digestion, and used for many investigations. It is known that casein micelles are formed by binding calcium phosphates to the phosphoryl residues of casein, and that soluble CPP–calcium phosphate complexes are formed by binding calcium phosphates to the phosphoryl residues of CPP, too. Therefore, CPP conjugated with calcium phosphate was prepared from casein micelles by tryptic digestion in the presence of calcium phosphates. The peptide composition of CPP from the casein micelles was compared with that of CPP from casein, and the calcium retention ability of both CPPs was then measured.

Materials and Methods

Chemicals. All chemicals were of the highest purity available, and were used without further purification.

Preparation of samples. Skim milk was prepared from bulk milk of Holstein cows by centrifugation at 2000 $\times$ g for 15 min. After sodium azide (to 0.02%) and e-aminoacaproic acid (to 0.1 mm) had been added to inhibit microbial growth and protease activity, respectively, the skim milk was stored at –30°C. When needed for use, the skim milk was thawed at 20 to 30°C.

Casein micelles were prepared from skim milk kept at 37°C for 2 h to allow the micelles to equilibrate. Casein micelles were obtained as a pellet from the skim milk by centrifuging at 100,000 $\times$ g for 1 h at 30°C. This pellet was dispersed into the ultratritration of the skim milk with a Potter-Elvehjem Homogenizer, the suspension being used as a casein micelle solution.

Acid-precipitated casein was prepared from skim milk by precipitating at pH 4.6 and 30°C, neutralizing to pH 7.0, dialyzing against deionized water, and lyophilizing. The lyophilized casein was dissolved in a 10 mm imidazole buffer (pH 7.0) and used as an acid-precipitated casein solution.

$\beta$-Casein was prepared from acid-precipitated casein by the method of Aschaffenburg, and purified by DEAE-Sephal chromatography (Pharmacia Biotech). CPP (4-CN-4P(1–25)) and CPPcn were prepared from $\beta$-casein and acid-precipitated casein, respectively, by the method of Peterson et al. The crude CPPs were purified by the method of Mann and Annan. $\beta$-CPP being further purified by DEAE-Sephal chromatography. CPPcm was prepared from casein micelles by tryptic digestion and gel filtration (described below).

An ultratritration was obtained from skim milk by using an ultratitration cell (model UHP-76, Advantec Toyo) with a UK-10 membrane filter (molecular weight cut-off at 10,000) at room temperature. A simulated milk ultratritration (SMUF) was prepared by the method of Jenness and Koops.

Tryptic treatment. Acid-precipitated casein and $\beta$-casein were dissolved in a 10 mm imidazole buffer (pH 7.0), and then digested by trypsin (Sigma Chemical Co.) at 25°C for 60 min. Since the urea-PAGE patterns of the tryptic caseins were not further changed by treating for more than 40 min, the digestion time was taken as 60 min.

Casein micelles were dispersed into the ultratritration of skim milk, and then digested by trypsin (Sigma Chemical Co.) at 25°C. The ratios of protein to enzyme for CPP preparation and particle size estimation were 300 and 1200, respectively. Trypsin inhibitor (from soybean, Sigma Chemical Co.) was added to terminate the reaction. Tryptic digestion for the CPP preparation was done at 25°C for 40 min from the gel filtration patterns of the digested casein micelles (described in the results section (Fig. 5)).

Particle size estimation. The average diameter of the casein micelles was estimated by the method of Holt et al., based on the wavelength dependence of turbidity. The particle size was obtained from the relationship between the size data and slope of the log(turbidity) against log(wavelength) plot in the range of 400 to 700 nm. The turbidity was measured by a Hitachi 200 spectrophotometer with the cell holder maintained at a definite temperature.

Ge chromatography. Toyopearl HW-55S gel chromatography (Tosoh)
was used to confirm the CPP–calcium phosphates complexes by using a pump with medium-flow rate (MPD-1L, GL Science) connected to a 1.0 × 60 cm column at room temperature. An imidazole buffer (10 mM, pH 7.0) containing 0.03 M NaCl, 22.5 mM CaCl₂, and 4 M urea was used as the elution solvent, urea being added to the buffer to dissociate the peptides. The elution profiles of the digested casein micelles varied with increasing urea concentration, and attained a definite pattern above 4 M urea. Therefore, 4 M urea was subsequently used for the elution buffer. Calcium of 22.5 mM was also added to the buffer to inhibit the liberation of calcium from the peptides. The eluent was collected by a fraction collector (Advantec Toyo), the peptides in the eluent being monitored with an ATTO UV monitor (model II) at 280 nm.

Molecular weight calibration. The molecular weights of the CPP–calcium phosphate complexes were estimated by comparing the retention times with those of standard proteins in a Toyopearl HW-55S column. T₅₀/T₉₀ values for standard proteins from gel chromatography were plotted against the logarithm of their molecular weights by the method of Whitaker,¹⁹ T₅₀ being the time required to elute the void volume of the column, and T₉₀ being the retention time for a standard protein. The following Standard proteins (Dalton Standard MS-II) were purchased from Serva Feinbiochemica: ferritin, ovalbumin, bovine serum albumin, and myoglobin; their respective molecular weights are 450,000, 67,000, 45,000, and 17,000.

Polyacrylamide gel electrophoresis (PAGE). Electrophoresis was performed in a vertical polyacrylamide slab gel, using a pH 7.5 buffer system containing 4 M urea and a modification of the method of Melachouris.¹⁹ The concentrations and pHs of the stacking and separation gels were 4 and 15%, and pH 5.9 and 7.3, respectively. Coomassie brilliant blue G-250 was used for peptide staining in the gel by the method of Blakesley and Boezi.²⁰ After electrophoresis, the gels were immersed in a staining solution containing 12% trichloroacetic acid for 12 h, the background stain in the gel then being removed with water.

Calcium binding ability of CPP. Calcium bound to CPP was measured according to the calcium content of the CPP–calcium phosphates (CPP) complexes. The CPP–CP complexes were formed by dissolving 0.5% of CPPcn or CPPcm in simulated milk filtrate (SMUF) and then stirring for 1 h at 37°C. The CPP–CP complex was separated by gel filtration through a Toyopearl HW-55S column.

Determination of peptide and protein. The peptide and protein contents of the solutions were determined by the method of Itzhaki and Gill.²¹ Before the determination, calcium was removed by centrifuging the samples after adding 2 N NaOH.

Determination of calcium and inorganic phosphorus. After the protein had been removed from the sample solutions by adding an equal volume of 20% trichloroacetic acid, calcium and inorganic phosphorus were determined by atomic absorption¹² and Nakamura's modification²³ of Allen's method, respectively.

Isolation of each CPP. CPPcn and CPPcm were chromatographed in a Q-Sepharose FF column (1 × 30 cm, Pharmacia Biotech) by eluting with a linear gradient of NaCl from 0.25 to 0.5 M in a 10 mM tris buffer (pH 7.0). The effluent was monitored by a UV detector (SPD-6AV, Shimadzu Corporation) at 220 nm. Each peak fraction was pooled and rechromatographed to obtain a single peak fraction, which was desalted by gel filtration in a Sephadex G-10 column (Pharmacia Biotech), and finally lyophilized.

Amino acid analysis. A CPP sample (200 µg) was dissolved in 1 ml of 6 N HCl, frozen at −80°C in an alcohol bath, and then degassed. The sample tube was next sealed, and incubated at 110°C for 24 h. Amino acids in the digested CPP were analyzed with a high performance amino acid analyzer (Hitachi model 853).

Results

Tryptic digestion of casein micelles

After adding trypsin, the casein micelle suspension was incubated at 25°C with the protein-to-enzyme ratio of 300. The turbidity was lost after about 15 min, the reaction being too rapid to estimate the micellar size. Therefore, a ratio of 1200 was used for monitoring the turbidity, which decreased with time and was lost after 80 min as shown in Fig. 1. The micelles were almost completely destroyed by the tryptic digestion for more than 80 min. The mean micellar diameter in the suspension, however, was constant until the turbidity was lost. This means that the digestion rate of the casein micelles was independent of the micellar size.

A protein-to-enzyme ratio of 300 was used for preparing CPP. Triptically digested casein micelles were eluted from a Toyopearl HW55S column with a 10 mM imidazole buffer containing 22.5 mM CaCl₂ and 4 M urea. The elution patterns for the digested casein micelles varied with digestion time from 0 to 30 min as shown in Fig. 2, and remained unchanged above 30 min. It seems that the tryptic digestion was completed after 30 min, so tryptic digestion was conducted for 40 min at 25°C to prepare CPP from the casein micelles.

Gel chromatography of the tryptic products of casein micelles and acid-precipitated casein

The tryptic products of casein micelles and acid-precipitated casein were examined using a Toyopearl HW55S column.

Fig. 1. Changes in the Micellar Diameter and Turbidity of a Casein Micelle Suspension for Various Times of Tryptic Digestion. Micellar casein was digested by trypsin at 25°C. The protein-to-enzyme ratio was 1200. ▲ is the micellar diameter, and ◦ is the turbidity at 400 nm.

Fig. 2. Changes in the Elution Patterns of Tryptically Digested Casein Micelles for Various Reaction Times. The tryptic products were eluted from a Toyopearl HW55S column with a 10 mM imidazole buffer (pH 7.0) containing 22.5 mM CaCl₂ and 4 M urea. a, b, c, d, e, and f as the times required for tryptic digestion are 10, 20, 30, 40, 50, and 60 min, respectively.
precipitated casein were chromatographed in a Toyopearl HW-55S column with a 10 mM imidazole buffer containing 22.5 mM CaCl2 and 4 M urea. The elution pattern of the tryptic casein micelles had 5 peaks (P0, P1, P2, P3, and P4) as shown in Fig. 3 (line b). P0 was eluted at the void volume. Therefore, fraction P0 seems to have been polymerized peptides or proteins not sufficiently dissolved. Since P1 was eluted before P2 to P4, the P1 fraction had a higher molecular weight than those of P2 to P4. On the other hand, the elution pattern of the tryptic acid-precipitated casein (line a in Fig. 3) has no P1. The peptide composition of these fractions was analyzed by urea PAGE, the PAGE pattern being shown in Fig. 4. The peptides in the P1 fraction from the tryptic casein micelles were electrophoresed as one band, and showed the same migration rate as that of β-CPP with 4 phosphoryl groups. The peptides in P1 had a high negative charge, similar to β-CPP. These peptides must have had phosphoryl groups, that is, CPP. The other fractions had no band at the expected position for β-CPP, so CPP is considered only to have been in the P1 fraction. The P1 fraction showed a higher molecular weight than the other peptide fractions in an imidazole buffer (pH 7.0) containing 22.5 mM CaCl2 and 4 M urea. It is known that calcium phosphates (CP) combined with the phosphoryl groups of caseins, form conjugated caseins by a CP-bridge and then made casein micelles,^{1,2} and that the CP-bridge is not broken in 4 M urea.^{20} It was thus expected that the P1 fraction contained CPP-CP complexes. On the other hand, acid-precipitated casein is prepared by removing CP, and does not contain a CP-bridge. The calcium and phosphorus contents of these fractions were measured and are shown in Table I. Calcium and inorganic phosphorus were contained mainly in the P1 fraction, the other fractions scarcely containing these salts. These results indicate that the peptides in the P1 fraction were CPP conjugated with CP, that is, CPP-CP complexes.

The P1 fraction was collected, and ethylenediaminetracetate·3Na was added to it. This fraction was dialyzed against deionized water using a Spectrum tube of molecular cut-off of 1000, and was freeze-dried. This freeze-dried P1 fraction contained 95 mg/g of casein. If the peptides in P1 were almost all CPP, the yield of CPP against the total of predicted CPPs from the primary structures of caseins would have been about 80%. The predicted major CPPs were \( x_{1}\)-CN·5P(f59–79), \( x_{2}\)-CN·4P(f1–25), \( x_{2}\)-CN·4P(f46–70), and \( x_{2}\)-CN·4P(f2–21),^{19} which are called \( x_{1}\)-CPP, \( x_{2}\)-CPP, \( x_{2}\)-CPPa, and \( x_{2}\)-CPPb, respectively, in this paper. The ratios of \( x_{1}\)-CN, \( x_{2}\)-CN, \( x_{2}\)-CN, and \( x\)-CN in whole casein are 3, 3, 0.8, and 1, respectively.

The organic phosphorus content of the freeze-dried peptide sample was measured as 60 mg of phosphorus/g of peptide. Assuming a molecular weight of 3000 for the

| Table I. Calcium and Inorganic Phosphorus Contents in the Peak Fractions Obtained from the Tryptic Products of Micellar and Acid-precipitated Caseins by Gel Filtration through a Toyopearl HW-55S Column |
|---|---|---|---|
| Tryptic product | Peak | Calcium | Inorganic phosphorus |
| from | P1 | 151 | 32.5 |
| casein | P2 | 38.1 | 14.8 |
| micelles | P4 | --- | --- |
| from | P1* | --- | --- |
| acid | P2 | 27.8 | 8.33 |
| casein | P4 | --- | --- |

* Fraction at peak position. mg/g of protein
peptides, they would be phosphopeptides having four to five phosphoryl groups per molecule.

The molecular weight of the CPP-CP complexes in fraction P1 was estimated by gel filtration chromatography. Logarithms of the molecular weights of standard proteins are proportional to the reciprocal of their elution times as shown in Fig. 5, the relational coefficient of this line being 0.998. The molecular weight of the CPP-CP complexes was estimated to be $170 \times 10^3$ from this linear relationship.

**Calcium binding abilities of CPPs obtained from acid-precipitated casein and casein micelles**

CPP-CP complexes were formed from CPPcm, CPPcn, or $\beta$-CPP by stirring at 37°C for 1 h in SMUF, and were eluted from a Toyopearl HW-55S column at 37°C as shown in Fig. 6. CPPcn-CP was eluted (line a) at the same position as that of $\beta$-CPP-CP (line b), while CPPcm-CP was eluted (line c) at an earlier position than that of $\beta$-CPP-CP. The molecular weights of these peaks were estimated by gel chromatography, that of CPPcm-CP being $101 \times 10^3$, which is five times the value for $\beta$-CPP-CP and CPPcn-CP ($18 \times 10^3$) as shown in Table II. The calcium and phosphorus contents of the complexes were measured by atomic absorption and Nakamura's methods, respectively. CPPcm-CP contained twice the quantity of calcium and phosphorus than $\beta$-CPP-CP and CPPcn-CP.

**Peptide composition of CPPs from casein micelles and acid-precipitated casein**

CPPcm and CPPcn were eluted as eight peaks (f0–7) from a Q-Sepharose FF column with a linear gradient of NaCl concentration from 0.25 to 0.5 M. The elution patterns are shown in Fig. 7. Fraction 4 of CPPcm is larger than that of CPPcn, and fraction 3 of CPPcn is larger than that of CPPcm. Fraction 0 is the unadsorbed fraction and contained

<table>
<thead>
<tr>
<th>Table II. Calcium and Inorganic Phosphorus Contents, and Molecular Weights of CPPcm-CP, CPPcn-CP, and $\beta$-CPP-CP</th>
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<tr>
<td><strong>CPPs</strong></td>
</tr>
<tr>
<td>CPPcm-CP</td>
</tr>
<tr>
<td>CPPcn-CP</td>
</tr>
<tr>
<td>$\beta$-CPP-CP</td>
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</table>

* mg/g of CPP

**Fig. 5. Relationship between the Elution Volume Ratio ($T_e/T_c$) and Molecular Weight (log MW) of Standard Proteins and P1 by Toyopearl HW-55S Chromatography.**

P1 is the peak in Fig. 3 (line b). FET, BSA, OVA, and MIG are ferritin, bovine serum albumin, ovalbumin, and myoglobin, the respective molecular weights being 450,000, 67,000, 45,000, and 17,000.

**Fig. 6. Elution Patterns for Complexes of CPPcn (a), $\beta$-CPP (b), and CPPcm (c) with Calcium Phosphate by Toyopearl HW-55S Chromatography.**

The CPP CP complexes were formed in a simulated milk ultrafiltrate (SMUF) by stirring the mixture for 1 hour at 37°C. The complexes were eluted with a 10 mM imidazole buffer (pH 7.0) containing 22.5 mM CaCl$_2$, and 4 mM urea.

**Fig. 7. Elution Patterns of CPPcn (a) and CPPcm (b) by Q-Sepharose FF Chromatography.**

The dotted line indicates the molarity of NaCl in a 10 mM tris buffer (pH 7.0).

**Table III. Amino Acid Compositions of the Fractions Obtained by Ion-exchange Chromatography of CPPcm in a Q-Sepharose FF Column**

<table>
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<tr>
<th>Fraction No.</th>
<th>f3</th>
<th>f4</th>
<th>f5</th>
<th>f6</th>
<th>f7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>1.1 (1)</td>
<td>1.3 (1)</td>
<td>3.4</td>
<td>2.0 (2)</td>
<td>3.1</td>
</tr>
<tr>
<td>Thr</td>
<td>1.2 (1)</td>
<td>0.4 (0)</td>
<td>1.0</td>
<td>1.0 (1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ser</td>
<td>4.8 (5)</td>
<td>5.1 (5)</td>
<td>8.9</td>
<td>4.0 (5)</td>
<td>4.7</td>
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<tr>
<td>Glu</td>
<td>7.1 (7)</td>
<td>8.4 (7)</td>
<td>8.3</td>
<td>8.0 (8)</td>
<td>8.9</td>
</tr>
<tr>
<td>Gly</td>
<td>1.0 (1)</td>
<td>0.4 (0)</td>
<td>1.2</td>
<td>1.1 (1)</td>
<td>1.0</td>
</tr>
<tr>
<td>Ala</td>
<td>0.1 (0)</td>
<td>0.8 (1)</td>
<td>2.5</td>
<td>2.5 (3)</td>
<td>1.6</td>
</tr>
<tr>
<td>Val</td>
<td>1.9 (2)</td>
<td>2.0 (2)</td>
<td>2.1</td>
<td>2.1 (2)</td>
<td>1.6</td>
</tr>
<tr>
<td>Ile</td>
<td>1.6 (2)</td>
<td>1.9 (2)</td>
<td>4.4</td>
<td>1.3 (1)</td>
<td>2.7</td>
</tr>
<tr>
<td>Leu</td>
<td>2.8 (3)</td>
<td>0.9 (0)</td>
<td>1.1</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
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<td>(0)</td>
<td>(0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>(0)</td>
<td>(0)</td>
<td>2.6</td>
<td>1.1 (1)</td>
<td>1.8</td>
</tr>
<tr>
<td>Arg</td>
<td>1.6 (2)</td>
<td>0.3 (0)</td>
<td>(0)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
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<td>1.3 (1)</td>
<td>2.4</td>
<td>(0)</td>
<td></td>
</tr>
</tbody>
</table>

**Assign-**

ment

$\beta$-CN-4P

$\alpha_1$-CN-5P

$\alpha_2$-CN-4P

( ) shows the numbers of amino acid residues of the known CPP.
a low amount of phosphorus, so that it may have contained the peptides beside CPP. Each fraction of 3 to 7 was rechromatographed, and single peaks were obtained from each fraction. The amino acid composition of each single peak fraction was analyzed, and is shown in Table III. The peptides originating from caseins (ζ₁₋₄, ζ₂₋₅, ζ₅, and ζ₆-casein) by tryptic digestion could be predicted from their known primary structures, the amino acid compositions of fractions 3, 4, and 6 agreeing with those of β-CN-4P(f₁₋₂₅), ζ₂₋₅-CN-5P(f₅₋₇₉), and ζ₅-CN-4P(f₆₋₇₀), respectively. The amino acid compositions of fractions 5 and 7 did not agree with any fragments from caseins by tryptic digestion.

Discussion

The mean diameter of casein micelles in the suspension was constant until the turbidity had been lost by tryptic digestion. This means that trypsin acted on both the surface and inside of the micelle. It is known that carboxypeptidase A can penetrate into the inside of a casein micelle. Therefore, it is considered that trypsin acts against the subunits of a casein micelle, the loss of micelles is independent of the micellar size.

Juillerat et al. reported that trypsin phosphopeptides obtained from whole bovine casein could be separated by chromatography in a Mono Q HR column, and identified these. CPPcm in our experiment was eluted as seven peaks from a Q-Sepharose FF column, the major peaks being identified and agreeing with the phosphopeptides obtained by the Mono Q column. The resin for Q-Sepharose FF is same as that for Mono Q (Pharmacia Biotech). CPPcm was also eluted in the same positions as those of CPPcm. The elution order of CPPcm and CPPcm by Q-Sepharose chromatography was same as that from the Mono Q column. The fractions not identified by Juillerat et al. were also observed on the Q-Sepharose column, and likewise could not be identified. The amino acid compositions of these fractions were not similar to any fragments predicted from the primary structure of caseins by trypsic digestion. It seems that these fractions were not single components, and/or that the peptides were formed by a protease other than trypsin. The major fractions (f₃ and f₄) of CPPcm were ζ₁₋₄-CPP and β-CPP, which contain 5 and 4 phosphates per molecule, respectively. The phosphorus content of CPPcm was 4.05 mol per 3000 g. The ratio of the other minor fractions in CPPcm (f₁, f₂, and f₅₋₇) calculated from the elution pattern (Fig. 7) was small (<30%).

CPPcm–CPP contained twice the quantities of calcium and phosphate than CPPcm–CPP (Table II). The major component of CPPcm was ζ₁₋₄-CPP containing 5 phosphatyl residues/molecule, and that of CPPcm was β-CPP containing 4 phosphatyl residues/molecule. The molecular weight of CPPcm–CPP was 5 times that of CPPcm–CPP. Aoki et al. have pointed out the incremental effect of one phosphatyl residues on the cross-linking of caseins by CP. The molecular weight of cross-linked human β-CN-4P by CP is reported to be 5.4 times that of human β-CN-3P, so it seems that ζ₁₋₄-CPP (containing 5 phosphatyl residues) plays an important role in solubilizing calcium phosphates.

Trypsin acts on the micellar subunits in a casein micelle suspension, and acts on various casein complexes in an acid-precipitated casein solution. It is known that micellar subunits consist of ζ₁₋₄-CN-κ-CN, ζ₂₋₅-CN-β-CN, and ζ₅-CN-κ-CN. The framework of the casein micelle is mainly made from ζ₁₋₄-CN. Trypsin can contact ζ₁₋₄-CN inside and on the surface of a casein micelle. On the other hand, protease in a casein solution is known to easily attack β-CN. CPP from the casein solution contained mainly β-CPP. CPPcm can be prepared as the precipitates of barium salts in an aqueous ethanol solution, and CPPcm can be prepared as the complexes conjugated with calcium phosphate in a urea solution. Since the framework of the casein micelle is mainly constructed from ζ₁₋₄-casein and colloidal calcium phosphates, CPPcm must contain a higher ratio of ζ₁₋₄-CPP, and have high retention ability for calcium phosphates.

References