Preparation and Characterization of Micellar Calcium Phosphate-Casein Phosphopeptide Complex

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Summary Micellar calcium phosphate (MCP) in bovine milk was separated as the complex with casein phosphopeptide (CPP) by the following procedures. Rennet curd obtained from skim milk was suspended in water, the pH was adjusted to 4.6, and the suspension was centrifuged at 1,000 × g. CPP was separated from the precipitated casein by tryptic hydrolysis and ethanol precipitation. The supernatant, which contained calcium and inorganic phosphate liberated from casein micelles by acidification, and CPP were mixed; the pH was adjusted to 6.7; and then the solution was lyophilized. From 1 L of skim milk, 3.16 g of the MCP-CPP complex was obtained. The dried MCP-CPP complex was easily dissolved in water and contained 12.7% calcium, 0.3% magnesium, 3.4% inorganic phosphorous, and 2.2% organic phosphorous. No crystal structure of hydroxyapatite was shown in the MCP-CPP complex by the X-ray diffraction analysis, although the pattern of NaCl crystal was observed. The X-ray diffraction pattern of commercial whey mineral, which was prepared by precipitation at alkaline pH from rennet whey, was similar to that of hydroxyapatite. It was confirmed by high-performance gel chromatographic analysis that the form of calcium phosphate in the MCP-CPP complex was similar to that of casein micelles. The MCP-CPP complex was also separated from commercial rennet casein. The method for the separation of MCP-CPP complex described above can be applied to the large-scale preparation.

Key Words calcium phosphate, phosphopeptide, casein micelle, rennet curd, hydroxyapatite

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Bovine milk is rich in calcium and superior in calcium absorption and bioavailability to other foods and inorganic calcium such as calcium carbonate (1, 2). Although lactose and phosphopeptides are considered to be factors enhancing calcium absorption (3–5), other reasons remain uncertain. In bovine milk, about two thirds of calcium and half of inorganic phosphate are present in casein micelles (6). Calcium phosphate in casein micelles is called micellar calcium phosphate (MCP) or colloidal calcium phosphate (7, 8). MCP binds to phosphate groups of caseins and cross-links casein molecules through their phosphate groups (9, 10). Calcium and inorganic phosphate in MCP are in quasi-equilibrium with those in soluble phase (6). It has been clarified that MCP is not a crystal, such as hydroxyapatite, but is amorphous (11). However, the structure of MCP has not been sufficiently resolved, although several models, such as an amorphous calcium phosphate model having a composition of Ca₉(PO₄)₆ by Schmidt (11), a burushite model by Holt et al (7), and an ion cluster model by van Dijk (8) have been proposed.

It is also suggested that calcium absorption is related to the chemical form in milk. Kato et al (12) reported that the bioavailability of calcium preparation from rennet casein was superior to that of the inorganic form of calcium. However, the calcium preparation from rennet casein was insoluble in water and showed the pattern of hydroxyapatite in X-ray diffraction analysis, probably because calcium and inorganic phosphate were liberated from rennet casein by acidification and dried.

To estimate the bioavailability of calcium in casein micelles, it is necessary that calcium be separated as a form as close as possible to that in casein micelles. Ono et al (13, 14) separated calcium phosphate-casein phosphopeptide (CPP) complex from casein micelles by the combination of tryptic digestion, disaggregation of the digested casein micelles with 6M urea, and gel-filtration or ultrafiltration. The MCP-CPP complex was also separated from commercial rennet casein (30g) after being suspended in 1L of deionized water.

MATERIALS AND METHODS

Materials. Raw skim milk was obtained from Kagoshima Milk Products. Commercial rennet casein (ALAREN771) and whey mineral (ALAMIN995) were purchased from the New Zealand Dairy Board. Tosyl-phenylalanine-chloromethyl-ketone-treated trypsin was purchased from Sigma Chemicals. Hydroxyapatite was from Seikagaku. Other chemicals used were guaranteed grade.

Preparation of MCP-CPP complex. The procedures used for the preparation of MCP-CPP complex are illustrated in Fig. 1. CPP was prepared as calcium salt by the method of Manson and Annan (16). The MCP-CPP complex was also separated from commercial rennet casein (30g) after being suspended in 1L of deionized water.
Preparation of Micellar Calcium Phosphate

Fig. 1. Procedures for the preparation of MCP-CPP complex from skim milk. MCP, micellar calcium phosphate; CPP, casein phosphopeptide.

Solubility test of MCP-CPP complex. The MCP-CPP complex was dissolved for the concentration of its organic phosphate to be 7 mM in water, and the solution was then centrifuged at 1,000 × g for 15 min at 20°C. Inorganic phosphate in the supernatant was determined. The solubility of the MCP-CPP complex was represented as the percent of inorganic phosphate in the supernatant to the total one.

Ion exchange high-performance liquid chromatography (IE-HPLC). This was performed with a TOSO CCPE chromatograph using a TSK-GEL DEAE-5PW column (7.5 × 75 mm) attached to a TSK guard column (6 × 10 mm). The calcium in the CPP fraction was removed by EDTA treatment followed by dialysis against deionized water. Calcium-free CPP was dissolved in 20mM NH₄HCO₃ (pH 8.0) containing 50mM NaCl (Buffer A) and applied to the column, then eluted with a linear gradient from 0 to 35% Buffer B for 10 min, from 35 to 50% Buffer B for 40 min, and from 50 to 100% Buffer B for 10 min at a flow rate of 0.5 mL/min. Buffer B contained 20mM NH₄HCO₃ (pH 8.0) containing 500mM NaCl. α₈₁-Casein-5P (f59-79) and β-casein-4P (f1-25) used as CPP standards were prepared.
from $\alpha_s$- and $\beta$-casein by the method of Manson and Annan (16), respectively, then purified by DEAE-cellulose column chromatography. $\alpha_s$- and $\beta$-casein were isolated by the methods of Zittle and Custer (17) and Aschaffenburg (18), respectively.

Gel permeation high-performance liquid chromatography (GP-HPLC). This was done with a Shimadzu LC-5A chromatograph using a TSK-GEL G3000SW column (7.5 mm × 60 cm) attached to a TSK guard column (7.5 mm × 7.5 cm). The elution buffer used was simulated milk ultrafiltrate (SMUF), which was prepared by using the method of Jenness and Koops (19). The reservoir of SMUF was cooled in an ice bath to prevent calcium phosphate precipitation, and analysis was performed at a room temperature below 25°C. The column temperature was maintained at 25°C. The MCP-CPP complex was dissolved in SMUF for the concentration of its organic phosphate to be 7 mM. To disaggregate the MCP-CPP complex, 5 mg of EDTA·$\text{Na}_2$·$\text{H}_2\text{O}$ and 8 mg of EDTA·$\text{Na}_4$·$\text{H}_2\text{O}$ were added to 1 mL of the MCP-CPP complex solution. The flow rate was 0.7 mL/min, and the injection volumes were 50 µL.

X-ray diffraction analysis. X-ray diffraction of the MCP-CPP complex was measured with a Rigaku Denki RU-200 diffractometer. Monochromatized CuKα radiation generated at 40 kV and 100 mA was used, and the count full scale was 3,000 c/s for all samples except NaCl (6,000 c/s). Casein micelles used for this analysis were separated from skim milk by ultracentrifugation at 100,000 × g at 25°C for 1 h, then lyophilized.

Chemical analysis. Calcium was determined with a Hitachi atomic absorption spectrophotometer. Inorganic phosphate and nitrogen were determined by the method of Allen and the micro-Kjeldahl method, respectively. Total phosphorous was determined after digestion with perchloric acid by the method of Allen (20). CPP was determined by the method of Miller (21). Organic phosphorus content was estimated from the difference between total and inorganic phosphorus contents.

RESULTS

MCP in bovine milk was separated as the complex with CPP according to the method shown in Fig. 1. From 1 L of skim milk, 3.16 g of MCP-CPP complex was obtained; its moisture content was 11.5%. The dried MCP-CPP complex was dissolved immediately after being put into deionized water, indicating excellent solubility in water. Table 1 shows the composition and solubility of MCP-CPP complex based on the dry basis. The ratio of calcium to inorganic phosphate of MCP-CPP complex from skim milk was 3.74, and it was slightly higher than the value of 3.05 for bovine casein micelles, which was calculated from the data reported by Schmidt (11). The high calcium content of the MCP-CPP complex was caused because CPP was separated as a calcium salt. CPP fraction contained 7.4% calcium, 0.9% inorganic phosphorus, and 3.9% organic phosphorus in its dry matter. When
Table 1. Composition and solubility of MCP-CPP complex.

| Starting material          | Composition (%) | Solubility in water (%) |
|---------------------------|-----------------|-------------------------|
|                           | N   | Ca   | Mg  | P_1 | P_o |         |
| Skim milk                 | 7.3 | 12.7 | 0.3 | 3.4 | 2.2 | 99.4     |
| Commercial rennet casein  | 6.9 | 13.8 | 0.4 | 3.3 | 2.1 | 99.2     |

Each data shows mean value of two determinations.

MCP, micellar calcium phosphate; CPP, casein phosphopeptide; P_1, inorganic phosphate; P_o, organic phosphate.

The MCP-CPP complex was put into water, it was easily dissolved in a few seconds, indicating an excellent solubility in water.

The IE-HPLC pattern of CPP from skim milk was shown in Fig. 2. The retention times of peaks 1 and 2 coincided with those of purified β-casein-4P (f1-25) and α51-casein-5P (f59-79), respectively. Reynolds et al (22) reported that the main components of CPP precipitated at pH 4.6 from tryptic digestion of whole casein by calcium and ethanol were β-casein-4P (f1-25) and α51-casein-5P (f59-79). Although CPP was prepared from skim milk treated with rennet in the present study, the major components of CPP seemed to be β-casein-4P (f1-25) and α51-casein-5P (f59-79).

The MCP-CPP complex was also prepared from the commercial rennet casein, which was coagulated without acidification and contained 11.5% moisture, 81.3% protein, 8.2% ash, and 2.8% calcium. The composition of the MCP-CPP complex from commercial rennet casein was closed to that from skim milk (Table 1).
Fig. 3. Gel permeation HPLC pattern of the MCP-CPP complex. Column, TSK-GEL G3000SW (7.5 mm × 60 cm); flow rate, 0.7 mL/min; elution buffer, simulated milk ultrafiltrate. a, MCP-CPP complex from skim milk; b, EDTA-treated sample a; c, MCP-CPP complex from commercial rennet casein; d, EDTA-treated sample c.

To characterize the MCP-CPP complex, GP-HPLC was performed by using SMUF as the effluent. As shown in Fig. 3, a and c, the eluate was divided into two fractions. Fraction 1 disappeared when EDTA was added to the sample solution (Fig. 3, b and d). Purified β-casein-4P (f1-25) and α₃-casein-5P (f59-79) both eluted at the same retention time as that of the fast eluted peak in fraction 2 in Fig. 3, a and c. We previously reported that caseins and egg white riboflavin-binding protein were cross-linked by calcium phosphate and that cross-linked proteins were disaggregated by the addition of EDTA (23, 24). Since EDTA solubilizes calcium phosphate, the addition of EDTA results in disaggregation of phosphoproteins cross-linked by calcium phosphate. The results of Fig. 3 suggested that CPP aggregates cross-linked by MCP, which was eluted in fraction 1, were shifted to fraction 2 by the addition of EDTA. Therefore it was considered that fraction 1 in Fig. 3, a and c was composed of CPP aggregates cross-linked by MCP.

To confirm hydroxyapatite in the prepared MCP-CPP complex, X-ray diffraction analysis was carried out. The X-ray diffraction pattern of hydroxyapatite used as a standard was shown in Fig. 4f. This pattern was almost the same as that reported by Matsushima et al (25). Although the peaks attributed to hydroxyapatite were not observed in the MCP-CPP complex, several peaks appeared
Fig. 4. X-ray diffraction pattern of the MCP-CPP complex, KCl, casein micelles, whey mineral, and hydroxyapatite (HAP). Count full scale, 3,000 c/s for samples a, c, d, e, and f, 6,000 c/s for sample b. a, MCP-CPP complex; b, NaCl; c, MCP-CPP complex concentrated by ultrafiltration; d, casein micelles; e, whey mineral; f, HAP.

(Fig. 4a). HCl and NaOH were used for the release of calcium and phosphate from the suspension of rennet curd and for the pH adjustment of the solution containing calcium, phosphate, and CPP (Fig. 1). Thus the possibility that NaCl crystal might be formed in drying the solution containing the MCP-CPP complex appeared. The X-ray diffraction pattern of NaCl was shown in Fig. 4b. The peaks observed in the diffraction pattern of MCP-CPP complex coincided in position with those of NaCl (Fig. 4, a and b). This clearly indicates that the dried MCP-CPP complex contains NaCl crystal. Subsequently, the solution containing calcium and inorganic phosphate liberated from casein micelles and CPP before lyophilization was concentrated to less than 1/10 volume by ultrafiltration and then lyophilized. As shown in Fig. 4c, the peaks in Fig. 4a were not observed in the pattern of the MCP-CPP complex concentrated by ultrafiltration. The pattern of Fig. 4c was very similar to that of amorphous calcium phosphate (25). Neither were steep peaks observed in the X-ray diffraction pattern of casein micelles (Fig. 4d). The diffraction pattern of commercial whey mineral, which was prepared from cheese whey by precipitation at alkaline pH, suggests the presence of a hydroxyapatite-like structure (Fig. 4e), although it was not as clear in comparison with that of hydroxyapatite (Fig. 4f). Consequently, it was concluded that no hydroxyapatite forms exist in the MCP-CPP complex prepared by the method shown in Fig. 1.
DISCUSSION

It has been considered that calcium in bovine milk is superior in bioavailability and is present in soluble and colloidal phases. A difference is found in the bioavailability of calcium between the two phases in bovine milk. Kato et al (12) reported that the calcium preparation from rennet casein, which corresponds to colloidal calcium, was well absorbed in comparison with calcium preparation from rennet whey. In the experiment, calcium and phosphate were liberated from the suspension of rennet casein by acidification and spray dried, and the prepared calcium sample showed the presence of hydroxyapatite by X-ray diffraction analysis. When the solutions containing calcium and phosphate were mixed, at first the precipitate of amorphous calcium phosphate was formed (11). The amorphous calcium phosphate was unstable and finally transformed to stable hydroxyapatite. The conversion of amorphous calcium phosphate to hydroxyapatite was prevented by casein, phosvitin, poly-L-glutamate, and so on (11, 26). In milk, the transformation of calcium phosphate to hydroxyapatite is prevented by casein. Calcium and phosphate in excess of their solubilities in milk are present as MCP binding to phosphate groups of caseins (9, 10). MCP is not present as a form of hydroxyapatite, but as an amorphous form, although its structure remains has not been sufficiently resolved (11). CPP also solubilizes calcium phosphate and may prevent the formation of hydroxyapatite. In the present study, therefore, MCP in bovine milk was separated as the complex with CPP.

The MCP-CPP complex was separated into two fractions, CPP cross-linked by MCP and CPP monomer by GP-HPLC (Fig. 3). Previously, we separated casein micelles into casein aggregates cross-linked by MCP and monomer of calcium caseinate by GP-HPLC, using SMUF containing 6 M urea as the eluate. The elution pattern of CPP cross-linked by MCP was similar to that of casein aggregates cross-linked by MCP. The X-ray analysis did not show the presence of hydroxyapatite in the MCP-CPP complex, and no pattern of hydroxyapatite was observed in casein micelles. These facts suggest that the form of calcium phosphate in the MCP-CPP complex is similar to that of casein micelles.

The MCP-CPP complex had excellent water solubility. Since all procedures used for the preparation of MCP-CPP complex were conventional ones, they were applicable to large scale-preparation. A high bioavailability of calcium was expected for the MCP-CPP complex. Experiments using animals on the calcium absorption are needed. The MCP-CPP complex may be a promising calcium source.

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