Note

Preparation of Casein Phosphopeptides from Casein Micelles by Ultrafiltration

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Casein phosphopeptides (CPPs), which inhibit the precipitation of calcium phosphate in the intestines, were prepared as CPP-calcium phosphate complexes from casein micelles by the ultrafiltration method. The prepared CPPs hardly contained any other peptides, so there was no need to treat with active carbon to remove the bitter peptides. The sum of $\alpha_s$-CN-5P(59–79) and $\beta$-CN4P(11–25) comprised 60% of the CPP composition by an analysis of the elution pattern from a Q-Sepharose FF column. The content of $\alpha_s$-CN-5P(59–79), which has high retention ability for calcium phosphate, was the highest in the CPPs.

Casein phosphopeptides (CPPs) are known to inhibit the precipitation of calcium phosphate in the intestines, and to increase the amount of soluble calcium available for absorption. CPPs are conventionally prepared from acid-precipitated casein by trypsin digestion, precipitation with barium or calcium ions, and then removal of the bitter peptides with active carbon. CPPs are used for food additives and in nutritional investigations. We have previously reported that CPPs from casein micelles as CPP–calcium phosphate complexes contained twice the quantity of calcium as CPPs from acid-precipitated casein. CPP from casein micelles contains more $\alpha_s$-CN-5P(59–79) than that from acid-casein and possesses a high retention ability for calcium phosphates. The preparation of CPPs from casein micelles was therefore examined.

It is known that CPPs from casein micelles can be obtained as complexes with calcium phosphate. The molecular weight of such casein complexes has been reported to be about $17 \times 10^4$ in milk-ultrafiltrate (MUF) containing 4 M urea. Therefore, CPP complexes are separable from other peptides by molecular size. The preparation of CPPs from casein micelles was examined by using ultrafiltration method, which can treat a large amount of a sample. The relative ratio of the peptide components of the CPPs was estimated by analyzing the elution pattern from Q-Sepharose FF chromatography.

Casein micelles, which are the materials used for CPP preparation, and milk ultrafiltrate (MUF) were prepared from skim milk as described in the previous paper. Simulated milk ultrafiltrate (SMUF) was prepared by the method of Jenness and Koops. Casein micelles were suspended in MUF and then digested by trypsin (Sigma) for definite times at 25°C. The ratio of protein to enzyme was 300. Since the activity of trypsin varies by lot, the incubation time was taken as 1.5 times the time required to reach a constant level of the soluble peptide. Trypsin inhibitor (twice the amount of the enzyme) and urea (to 4 M) were then added to terminate the reaction and to solubilize the peptides, respectively. The urea was used as a specially prepared reagent for biochemical studies (Nacalai), and the trypsin inhibitor (from soybean) was purchased from Miles. The peptide and protein contents of each solution were determined by the method of Izhaki and Gill.

The tryptic product of the casein micelles was filtered through an AHP-0013 ultrafiltration membrane (M.W. cut-off at 50,000) with an pencil-type module (Asahi Kasei). Membrane filtration can treat a greater amount of a sample in a shorter time than gel filtration can. MUF containing 4 M urea was then added to the residue, and the resulting solution was filtered again by the same procedure. This process was repeated three times to thoroughly separate the other peptides, the required time for filtration being about 4 h per 300 ml of sample. The residue was analyzed by urea-polyacrylamide gel electrophoresis (urea-PAGE) containing 1% mercaptoethanol, which was performed in a vertical slab gel, using a pH 7.5 buffer system containing 4 M urea by a modification of the method of Williams and Reisfeld. Details of this procedure have been described in a previous paper. The PAGE pattern of the residue shows a main narrow band and a minor broad band in Fig. 1-D. The main band is considered to have been CPP because its mobility was the same as that of $\beta$-CPP (Fig. 1-C). The minor band seems to have been from an aggregate of peptides because of the residues by ultrafiltration at a molecular weight cut-off of 50,000.

The peptide aggregate must be removed from the residue to purify CPP. When a calcium-chelating agent is added, CPP will exist in a free form in the calcium phosphate complex and the peptide aggregates will remain as themselves. Ethylenediamine tetraacetate-3Na (to 30 mm) was therefore added to the residue, which was then filtered through an ultrafiltration membrane with a molecular weight cut-off of 10,000. The filtrate will contain CPP. The PAGE patterns of the residue (Fig. 1-E) and of the filtrate

![Image](image_url)

Fig. 1. Urea-Polyacrylamide Gel Electrophoresis (pH 7.0) of the Fractions Obtained from Tryptic Products of Casein Micelles by Ultrafiltration.
A, casein; B, tryptic product of casein micelles; C, $\beta$-CPP; D, residue from the tryptic product by ultrafiltration through a membrane filter with a molecular weight cut-off at 30,000; E and F, residue and filtrate from the above residue in the presence of EDTA-3Na after ultrafiltration through a membrane filter with a molecular weight cut-off at 10,000, respectively.

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Table Composition of CPPs Prepared from Casein Micelles Separated with a Q-Sepharose FF Column

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Composition (%)</th>
<th>Estimated origin</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>14.2</td>
<td>Mixtures of F2 to F7</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>Unknown</td>
</tr>
<tr>
<td>3</td>
<td>30.7</td>
<td>β-CN-4P (f1-25)</td>
</tr>
<tr>
<td>4</td>
<td>31.9</td>
<td>x12-CN-5P (f59-79)</td>
</tr>
<tr>
<td>5</td>
<td>8.3</td>
<td>Unknown</td>
</tr>
<tr>
<td>6</td>
<td>3.5</td>
<td>x12-CN-4P (f46-70)</td>
</tr>
<tr>
<td>7</td>
<td>5.8</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

The absorbance at 220 nm is compatible to the peptide concentration, and the elution pattern was resolved into a set of Gaussian curves (the solid lines in Fig. 2) by minimizing the difference between the sum of the Gaussian curve areas and the area of the elution curve. The calculations were performed by the damped least-squares method with a PC-9801 FA personal computer (NEC). The composition of each peak (%) calculated from the peak area against the total area is shown in the Table, the sum of β-CN-4P (f1-25) and x12-CN-5P (f59-79) amounting to 62.6%. The content of x3-CN-5P (f59-79) was 32%, being the largest component in CPP. CPP from casein micelles is known to contain more x3-CN-5P (f59-79) than that from acid-casein and to possess a high retention ability for calcium phosphates.

This method could prepare CPPs as the CPP-CP complexes by using an ultrafiltration instrument (follow-fiber type) and without needing the addition of barium or calcium ions. The resulting CPPs possessed a higher retention ability for calcium phosphates than that from acid-casein. Since these CPPs hardly contained any other peptides, it was not necessary to treat with active carbon to remove the bitter peptides. However, separation of the CPP-CP complexes was done in a 4 M urea solution, and the urea had to be removed by dialyzing with a membrane.

References