Effect of Bound Phosphate on the Calcium-binding Ability and Calcium-dependent Precipitability of Human \( \beta \)-Casein

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The calcium-binding ability and calcium-dependent precipitability of human \( \beta \)-casein were studied and compared regarding components with different phosphorus contents. The calcium-binding ability and phosphorus content showed a linear correlation except for the component carrying no phosphate (0-P component). Calcium-dependent precipitability was also related to the phosphorus content, the components with more than 3 phosphates coagulating rapidly in the presence of 20 mM calcium, while the other components were quite stable even in this solution. However, solutions of the 0-P and 1-P components were turbid in the absence of calcium, because of strong hydrophobic interactions. When the 1-P and 5-P components were mixed in the presence of calcium, the 5-P component was protected from calcium-dependent precipitation. The 2-P component also showed a little stabilizing activity, but the 0-P component did not. These results suggest that the formation of human casein micelles may be quite complicated.

It is known that the major component of human casein constituents is \( \beta \)-casein, and the human casein micelle is thought to be constituted from \( \beta \)- and \( \kappa \)-caseins and calcium ions. The possibility of the presence of the component corresponding to \( \alpha_s \)-casein has been suggested by means of electrophoresis.\(^{1,2}\) Recently, a minor human casein component was found by Yoshikawa and Chiba,\(^{3}\) and was demonstrated to have a homologous amino acid sequence to that of bovine \( \alpha_s \)-casein in the N-terminal region. Human \( \beta \)-casein consists of 6 components which have the same polypeptide but possess different phosphorus contents.\(^{4-7}\) This has recently been confirmed by the completion of its amino acid sequence by Greenberg et al.\(^{8}\) The ratios of the differently phosphorylated components vary among the milks of different individuals,\(^{9,10}\) and this has been ascribed to a genetic control mechanism.\(^{11}\)

The significance of multi-phosphorylation in the biosynthesis of \( \beta \)-casein, in the formation of casein micelles or in infant nutrition is still unknown, but the physicochemical properties of individual human \( \beta \)-casein components may differ not only from those of bovine \( \beta \)-casein, which uniformly has 5 phosphoseryl residues, but also from other components of human \( \beta \)-casein.

In the previous paper,\(^{11}\) we showed, using isolated human \( \kappa \)-casein and \( \beta \)-casein components that were separated according to their phosphorus content, that highly phosphorylated human \( \beta \)-casein components were preferable for micelle formation and that the 0-P component did not make micelles with \( \kappa \)-casein. In the present paper, the differences in calcium-binding ability, calcium-induced precipitation and hydrophobicity were studied and compared among the 6 components. The effect of components containing low phosphorus on the stability to calcium of mixtures consisting of components with high and low phosphorus contents were also studied.

Materials and Methods

\( Human \ \beta \)-casein. Human whole casein was prepared according to the method of Nagasawa et al.\(^{12}\) from mature milk given by a healthy mother one month after parturition. After gel filtration of the whole casein on a
Sephacryl S-300 column (2.0 × 97 cm) equilibrated with 50 mM imidazole-HCl buffer (pH 7.0) containing 4 M urea and 0.01% 2-mercaptoethanol, the β-casein fraction was further fractionated into 6 components on DEAE-Sephacel, according to the phosphorus content. The β-casein fraction obtained by gel filtration was dialyzed against deionized water and lyophilized. Two hundred mg of the dry material was dissolved in 5 ml of imidazole-HCl buffer (pH 6.5) containing 4 M urea and 0.01% 2-mercaptoethanol, and was then applied to a DEAE-Sephacel column (1.6 × 15 cm), which has been equilibrated with the same buffer. Elution was carried out at 4°C with a linear gradient of NaCl (0~0.1 M). The break-through fraction (containing the 0-P component) was further purified on DEAE-Sephacel using the imidazole-HCl buffer, but at pH 7.5, containing 4 M urea and 0.01% 2-mercaptoethanol.

Bovine β-casein. Bovine β-casein was prepared by the method of Aschaffenburg13) and further purified using hydrophobic interaction chromatography on Octyl-Sepharose CL-4B according to the method of Creamer and Matheson.14)

Calcium-binding ability. The calcium-binding ability of each human β-casein component was estimated by using the centrifugation method described by Yoshikawa et al.15) Each casein component (5 mg) was dissolved in 1 ml of imidazole-HCl buffer (pH 7.1) containing 70 mM KCl and 0.02% NaN₃ in the presence of 20 mM CaCl₂ in ice-cooled water. The solution was then warmed and left to stand for 1 hr at 37°C, and was centrifuged at 8,500 x g for 1 hr. Calcium bound to the precipitated β-casein was estimated from the decrease in calcium and protein concentrations of the supernatant. Protein concentration was calculated from the absorbance at 280 nm after dilution with 50 mM potassium citrate (E° = 7.3). Calcium concentration was measured by inductively coupled plasma atomic emission spectroscopy.

Calcium-dependent precipitation. Each β-casein component was dissolved in 10 mM imidazole-HCl buffer, pH 7.1, containing 70 mM KCl and 0.02% NaN₃ in the presence of 20 mM CaCl₂. The solution was prepared in ice-cooled water to avoid the polymerization of β-casein and then transferred to a cell which had been maintained at 37°C in a spectrophotometer. The formation of β-casein coagula was evaluated by measuring the time course of the development of turbidity at 320 nm.

Stability of the β-casein mixture consisting of components with low and high phosphorus contents in the presence of calcium. The 4-P or 5-P component of human β-casein was dissolved in 10 mM imidazole-HCl buffer, pH 7.1, containing 70 mM KCl to make 0.6% solutions. The 0-P, 1-P and 2-P components were dissolved in the same buffer to make 3% solutions. The 4-P or 5-P components and the low phosphorus components were mixed to give the ratios of the low P component/4, 5-P component from 0.025 to 0.4 in ice-cooled water, and the final volume was adjusted to 0.9 ml with the same buffer. After the mixture had been left to stand for 30 min at 4°C, 0.1 ml of 200 mM CaCl₂ solution was added, and the mixture was incubated for 1 hr at 37°C before centrifugation at 1,000 × g for 1 min at 37°C. To 0.4 ml of the supernatant was added 1 ml of 50 mM potassium citrate solution. The concentration of β-casein remaining in the supernatant was calculated by measuring the absorbance at 280 nm.

Effect of the phosphate on hydrophobicity. The difference in hydrophobicity among the 6 components was estimated by means of HPLC, i.e., hydrophobicity was estimated from the retention time of each component eluted from a reverse-phase column. Each component was dissolved in 4 M urea (0.02%, w/v) and applied to an SSC-SC4 column (Senshu Kagaku) using a Jasco Trirotor SR-2 HPLC. Elution was carried out at room temperature with 5 mM phosphate buffer (pH 7.0) and a linear gradient of acetonitrile from 34% to 40% in 30 min. The flow rate was 1 ml/min.

Results and Discussion

Figure 1 shows the relationship between the phosphorus content and the amount of calcium bound to β-casein, measured by the centrifugation method already described. By this method, the binding of calcium to bovine β-casein was calculated to be 5.6 mol/monomer, which agrees with the result of Yoshikawa et al.15) Prior to the trial of this method, the dialysis-equilibrium method and gel filtration method16) had been attempted to...
estimate the calcium-binding ability of human β-casein. However, at above 10°C, calcium-dependent precipitation of β-casein occurred, which made these attempts unsuccessful.

The calcium-binding ability of human β-casein was highly correlated to the phosphorus content, except for the 0-P component, which showed abnormal behavior as if it possessed high binding ability. By the centrifugation method, the amount of calcium bound to this component was estimated to be 6.2 mol/monomer, which corresponded to that of the 4-P component. It has been reported by Yoshikawa et al. that, in the case of bovine β-casein, the calcium-binding ability decreased according to the degree of dephosphorylation (95% dephosphorylation reduced the amount of bound calcium to 0.4 mol/monomer) and the calcium-dependent precipitation was completely lost by 65% dephosphorylation. We do not have enough data at the present time to explain the abnormal behavior of the 0-P component of human β-casein, and further investigations are necessary to clarify the unusual properties of this component.

Figure 2 shows the calcium-dependent precipitation of human β-casein components, evaluated from the increase of turbidity at 320 nm and 37°C. The components with 0~2 phosphates gradually became turbid as time progressed; while the others swiftly reached maximum turbidity. During incubation, flocculation of the highly phosphorylated components occurred, and consequently, their turbidity began to decrease within 10 min.

According to the report of Greenberg et al., human β-casein components with more than 3 phosphates have the 3-continuous phosphoseryl sequence (Ser8–Ser10). This series of 3 phosphoseryl residues may have great influence on the conformation and self-association of β-casein owing to the considerable negative charge of the phosphate, i.e., in the presence of calcium, Ca^{2+} ions bind to the series of phosphate and the bound calcium causes calcium-dependent precipitation of β-casein, while, in the absence of calcium, the negative charge of the phosphate cluster prevents β-casein associating with itself. In the 4 and 5-P components, phosphates bind to Thr3 and/or Ser6 as well as to Ser8–Ser10, but these additional phosphorylated sites may have less influence than the 3 serial phosphates.

In Table 1, the self-association of each human β-casein component in the absence of calcium is compared, being evaluated from the turbidity at 320 nm and 37°C. The 0-P and 1-P components became turbid while the others remained unchanged. The increase in turbidity of 0-P and 1-P components is thought to be caused by the self-association of these components through hydrophobic binding, be-

![Fig. 2. Calcium-dependent Precipitation of Human β-Casein Components.](image)

<table>
<thead>
<tr>
<th>Component</th>
<th>Turbidity at 320 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-P</td>
<td>0.382</td>
</tr>
<tr>
<td>1-P</td>
<td>0.249</td>
</tr>
<tr>
<td>2-P</td>
<td>0.033</td>
</tr>
<tr>
<td>3-P</td>
<td>0.041</td>
</tr>
<tr>
<td>4-P</td>
<td>0.034</td>
</tr>
<tr>
<td>5-P</td>
<td>0.052</td>
</tr>
</tbody>
</table>

Table 1. Self-Association of Human β-Casein Components in the Absence of Calcium

The turbidity of each sample was measured after a 60-min incubation at 37°C.
cause the average hydrophobicity estimated by Bigelow's calculation method\textsuperscript{18} shows that the hydrophobicity of the 0-P component was much higher than that of dephosphorylated bovine \(\beta\)-casein, which possesses quite strong hydrophobicity (6.23 kJ/residue for the 0-P component and 5.58 kJ/residue for bovine dephosphorylated \(\beta\)-casein). In the next experiment, we investigated the difference in hydrophobicity between the 6 components of human \(\beta\)-casein by means of reverse-phase HPLC. Figure 3 shows the relationship between the phosphorus content and the retention time of human \(\beta\)-casein components on the SSC-SC column, that of bovine \(\beta\)-casein also being shown as a comparison. In the system applied here, the retention time decreased linearly with the degree of phosphorylation. On the other hand, bovine \(\beta\)-casein broke through the column under the conditions used. The results represent the difference in hydrophobicity between human and bovine \(\beta\)-caseins, and the marked effect of the phosphoserine residue on the hydrophobicity of human \(\beta\)-casein. It is likely that the multi-phosphorylation and strong hydrophobicity of human \(\beta\)-casein makes its physicochemical properties differ considerably from those of bovine \(\beta\)-casein. It is also possible that the behavior in casein micelle formation is different between human and bovine \(\beta\)-caseins and, furthermore, between each component of human \(\beta\)-casein. They may play different roles in micelle formation according to their degree of phosphorylation.

When each component of human \(\beta\)-casein was individually incubated at 37°C in the presence of calcium, highly phosphorylated components (3 to 5-P) could not remain stable in the supernatant, forming precipitates after a long-term incubation, while the less-phosphorylated components remained quite stable in the supernatant, even when the solution became turbid, because of the coagulum formation by self-association through the intermediary of \(\text{Ca}^{2+}\) (Fig. 2). Taking account of these facts, the stability of the coagula formed by mutual association of the different components was studied in the presence of calcium. Figure 4 shows the stability of the mixture consisting of the 4-P or 5-P component and the 0-P, 1-P or 2-P component, expressed as the percentage of protein remaining in the supernatant. The 1-P component markedly stabilized the 5-P component against precipitation; the coagulum seemed to form a colloidal dispersion. The 2-P component slightly stabilized the 5-P component when the ratio of 2-P/5-P was high, but the 0-P component seemed to stimulate precipitation rather than stabilize the 5-P component.
Properties of Human $\beta$-Casein Components

It has been demonstrated that dephosphorylated bovine $\beta$-casein stabilized $\alpha_\text{S1}$-casein against precipitation at 35°C in the presence of calcium, as does $\kappa$-casein.$^{19}$ The stabilizing activity of dephosphorylated $\beta$-casein has been ascribed to its solubility at physiological temperature, as well as to its amphiphilic structure, because native $\beta$-casein also protected $\alpha_\text{S1}$-casein from precipitation at low temperature. In the case of the human casein micelle, it may be possible that, at physiological temperatures, the highly phosphorylated $\beta$-casein components act as bovine $\alpha_\text{S1}$-casein does, and the less-phosphorylated components stabilize them similarly to the stabilization of $\alpha_\text{S1}$-casein by dephosphorylated bovine $\beta$-casein. However, it was not the 0-P component but the 1-P component that stabilized the 5-P component in the presence of calcium.

It has been accepted, following the report of Hill and Wake,$^{20}$ that the amphiphilic structure of $\kappa$-casein plays an important role in its ability to stabilize micelles. Through studies on the effect of the chemical phosphorylation of bovine $\kappa$-casein on its stabilizing ability, another factor for $\kappa$-casein as a stabilizer has been proposed by Yoshikawa et al.$^{15}$ i.e., its phosphorus content must be low. Prior to this postulation, it had been reported that stabilizing ability of bovine $\kappa$-casein was not affected by dephosphorylation.$^{21}$ The human $\beta$-casein 1-P component seems to satisfy the requirements for a stabilizer, but the 0-P component did not have stabilizing ability. It seems that human $\beta$-casein cannot be amphiphilic without phosphate because of its high hydrophobicity.

Therefore, we believe that it is possible that, despite the lack of $\alpha_\text{S1}$-casein in human milk, the multi-phosphorylation of $\beta$-casein makes the structure of the human casein micelle complicated and quite different from that of the bovine casein micelle, models of which have been proposed by several investigators.$^{22-25}$

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References