Cold-disaggregation of the Casein Micelles in Heated Concentrated Whey Protein-free Milk

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The amount of the soluble casein formed by cooling was much larger in the concentrated whey protein-free milk (WPF milk) heated at 135 - 140°C for 15 sec than in the unheated one. In the case of the heated concentrated WPF milk, the amount of soluble casein increased remarkably during the first 1 hr of cooling and gradually thereafter, while in the case of the unheated one the equilibrium between soluble and micellar casein was established after 1 hr of cooling. The temperature-dependent conversion of micellar casein to soluble casein was reversible in both the heated and the unheated concentrated WPF milk. The soluble casein formed by cooling bound a small amount of calcium. The κ- and ω-casein contents of the soluble casein formed by cooling the heated concentrated WPF milk were somewhat higher than those of the soluble casein formed by cooling the unheated one.

It is considered from the above results that the structure of casein micelles is loosened by heat treatment of concentrated milk.

It is well known that heating milk at high temperature causes aggregation of casein micelles. Recently, however, a few investigators pointed out that heating milk caused also disaggregation of casein micelles, namely, formation of soluble casein. In heated milk, a part of the heat-denatured whey proteins remains in the supernatant after ultracentrifugation and precipitates at the isoelectric point of casein. Such heat-denatured whey proteins cannot be distinguished from soluble casein by any of the common nitrogen distribution determinations. Accordingly, using whey protein-free milk (WPF milk), we confirmed the formation of soluble casein by heating. The formation of soluble casein occurs above 105°C and the amount of the soluble casein is proportional to heating time. Concentrating milk accelerates the formation of soluble casein by heating. The soluble casein formed by heating contains a large proportion of κ-casein. Rose demonstrated that β-casein constituted about 55% of the total increase in the soluble casein obtained by storing milk overnight at 4°C.

It is of interest to compare the cold-disaggregation of the casein micelles in the heated concentrated WPF milk with that in the unheated one. The aim of the present study is to provide further informations on the properties of the casein micelles in a heated concentrated milk system.

MATERIALS AND METHODS

Preparation of WPF milk and heat treatment of milk sample. WPF milk was prepared according to the method described in the previous paper. The outline is as follows. Skim milk was prepared from raw herd milk of about 60 cows by centrifugation without warming and then ultracentrifuged at 78,000 × g for 1 hr at 20°C. The ultrafiltration of the ultracentrifugal supernatant was carried out with a Diaflo membrane filter UM-10. The ultracentrifuged casein micelles were crushed in a mortar and suspended in the ultrafiltrate. The suspension was treated with ultrasonics of 9 kHz for 30 min at about 20°C. The final casein concentration in the WPF milk was adjusted to 2.5% by addition of the ultrafiltrate.
The WPF milk was concentrated to 1/2.5 of the original volume with a rotary evaporator below 35°C. The concentrated WPF milk was sealed into many glass tubes having 4 mm of diameter. Then, the glass tubes were immersed in an oil bath at 140°C for 1 min. Under the above heating conditions, the inner milk sample was estimated to be heated at 135-140°C for 15 sec.

Cooling of milk sample. Milk sample was cooled in cold water at 5°C for about 5 min, and then allowed to stand in a cold room at 5°C.

Determination of soluble casein. Milk sample was ultracentrifuged at 45,000 rpm (109,800 x g) for 1 hr at 5°C or 25°C with a Hitachi 55-P type ultracentrifuge. To 1 ml of the ultracentrifugal supernatant, 3 ml of 1/10 M acetate buffer (pH 4.4) was added. It was held for 15 min at 35°C. After filtration through a filter paper, nitrogen in the filtrate was determined by the Kjeldahl method and regarded as the non-casein nitrogen (NCN). The amount of soluble casein was calculated by multiplying the difference between the total nitrogen in the ultracentrifugal supernatant and NCN by 6.38.

Gel filtration. Gel filtration was carried out at 5°C on a column (2.6 x 45 cm) of Sephadex G-100. The gel column was prepared by pouring the gel particles in distilled water. Five ml of the ultracentrifugal supernatant was applied to the bottom of the column. The column was eluted with distilled water and the flow rate was about 25 ml/hr. The effluent was collected in 5 ml fractions. The void volume of the column was distilled about 100 ml.

Preparation of casein samples. Soluble casein was precipitated from the ultracentrifugal supernatant at pH 4.6, dissolved in water by the gradual addition of 1/10 N sodium hydroxide with caution that the pH value would not exceed 7, and then purified by reprecipitation at pH 4.6 followed by successive washing with water, ethyl alcohol, and acetone.

Whole casein was precipitated from WPF milk at pH 4.6, and then purified in the same manner as in the soluble casein.

ε-Casein was isolated from acid-precipitated casein obtained from skim milk by a modification of the method of Zittle and Custer. The net casein content in the samples was determined by the Kjeldahl method.

Calcium, inorganic phosphorus, and sialic acid. Calcium was determined with a Hitachi 207 atomic absorption spectrophotometer on the solution containing the filtrate of 1:1 mixture of the effluent and 24% trichloroacetic acid (TCA), 500 ppm of strontium, and 20% ethyl alcohol. Inorganic phosphorus was determined on the TCA filtrate by Allen's method. Sialic acid was determined by a modification of Warren's method.

Disc-gel electrophoresis. Disc-gel electrophoresis was performed with 7% acrylamide-gel containing 4.5 M urea by a modification of the method of Groves and Kiddy. Casein samples were dissolved in 6.6 M urea solution containing 0.3% 2-mercaptoethanol. They were then allowed to stand overnight. For each run, 3 µl of the 6.7% casein solution was employed. In order to prevent reoxidation of reduced ε-casein during electrophoresis, the separating gels allowed to stand overnight after preparation were used. Electrophoresis was performed at a constant current of 2 mA per column for about 3 hr at 5°C. Gels were stained with 0.3% amido black in 7% acetic acid solution for 1 hr and then destained by means of repeated washing with 7% acetic acid.

DEAE-cellulose column chromatography. By the method of Woychik and Kalan, 250 mg of casein sample was alkylated. DEAE-cellulose column chromatography was carried out according to the method of Rose et al. To a column (1.5 x 30 cm) of DEAE-cellulose (0.9 meq/g, Brown Co) equilibrated with tris-citrate-urea (TCU) buffer (pH 8.6, 0.005 M tris, 6 M urea), was added 20 ml of the alkylated casein solution. After washing with 50 ml of TCU buffer, elution was carried out with the same buffer with a linear gradient of sodium chloride from 0 to 0.3 M. A fraction volume was 10 ml.

The protein content was calculated from the absorbance at 280 nm. The absorbivities (E1%1cm, 280nm) used were 9.2 for αs-casein, 7.1 for β-casein, 8.5 for ε-casein, 9.2 for Fraction I which is composed of minor casein components such as TS- and γ-casein.

RESULTS

Effects of cooling and warming on the amount of soluble casein

The concentrated WPF milk heated at 135-140°C for 15 sec and the unheated one were cooled at 5°C for 1, 2, 4, 8, and 20 hr, and the amount of soluble casein was determined. As shown in Fig. 1, in the unheated concentrated WPF milk, the equilibrium between soluble and micellar casein was established after 1 hr of cooling; the amount of soluble casein had increased within 1 hr. This increasing tendency differed a little from that in an unconcentrated milk system shown by Watanabe et al. They reported that the equilibrium
The initial concentration of casein was 6.25 g in 100 ml of the concentrated WPF milk. Milk samples were cooled at 5°C. ○–○, control (milk samples were ultracentrifuged at 25°C). ●–●, heated concentrated WPF milk; ○–○, unheated one.

was established after 4 hr of cooling. In the case of the heated concentrated WPF milk, both the increasing tendency of soluble casein and the increment differed from those in the unheated concentrated WPF milk. The amount of soluble casein increased remarkably during the first 1 hr of cooling and gradually thereafter (Fig. 1). The increments of soluble casein in the heated concentrated WPF milk after 1 and 20 hr were about 2.5 and 4 times those in the unheated one, respectively.

Figure 2 shows the effects of warming on the aggregation of the soluble casein formed by cooling. The concentrated WPF milk cooled for 24 hr at 5°C was warmed for 4 hr at 25°C. In both the heated and the unheated concentrated WPF milk, the amount of soluble casein decreased to the level before cooling (Fig. 2, No. 3). The conversion of micellar casein to soluble casein was thoroughly reversible.

Only the ultracentrifugal supernatant from the concentrated WPF milk cooled for 24 hr at 5°C was warmed. The amount of soluble casein decreased to the level before cooling in the case of the unheated concentrated WPF milk (Fig. 2, white column, No. 4). In the case of the heated concentrated WPF milk, however, the amount of soluble casein did not decrease to the level before cooling (Fig. 2, black column, No. 4). In the heated concentrated WPF milk, a part of the soluble casein formed by cooling did not aggregate in the absence of casein micelles.

**Gel filtration of the ultracentrifugal supernatant from the cooled heated concentrated WPF milk**

The gel filtration diagram of the ultracentrifugal supernatant from the heated concentrated WPF milk cooled for 24 hr at 5°C is shown in Fig. 3. The first and second eluted fraction (Fractions I and II) contained casein. The third eluted fraction (Fraction III) contained no casein. Fraction III seems to be composed of non-protein substances which exhibit the absorption at 280 nm. Although Fraction I was slightly turbid, the absorbance was represented without correction. The ratio of casein content in Fractions I and II was about...
The patterns of disc-gel electrophoresis of the casein in Fractions I and II are also shown in Fig. 3. The pattern of the casein shows a larger zone of $\beta$-casein and a smaller zone of $\alpha_s$-casein in Fraction II than those in Fraction I. As described later, the soluble casein formed by cooling contained a large proportion of $\beta$-casein (Fig. 4). A large portion of the casein in Fraction II seems to be composed of the soluble casein formed by cooling. Fraction II contained a small amount of calcium but no inorganic phosphorus. Fraction I contained a slight amount of calcium. As reported in the previous paper, in the case of the unheated heated concentrated WPF milk, no calcium was found in the fraction containing casein. Accordingly, the soluble casein formed by cooling seems to bind a small amount of calcium. Almost all of inorganic phosphorus was found in Fraction III.

**Composition of soluble caseins**

Figure 4 shows the patterns of disc-gel electrophoresis of soluble casein and whole casein. The zone of $\kappa$-casein of the soluble casein from the heated concentrated WPF milk was stained deeper in comparison with that of whole casein, but the zone of $\alpha_s$-casein of the soluble casein was smaller than that of whole casein (Fig. 4, Nos. 1 and 2). When the heated and the unheated concentrated WPF milk were cooled, the zone of $\beta$-casein of the soluble casein became larger and the zone of $\alpha_s$-casein smaller (Fig. 4, Nos. 3 and 4). The zone of $\beta$-casein of the soluble casein from the cooled heated concentrated WPF milk was smaller than that of the cooled unheated one, but on the contrary the zone of $\alpha_s$-casein was larger. Since the reproducibility of the determination of casein components by
densitometry was poor, the composition of casein components was not shown.  
\( \kappa \)-Casein plays the most important role for the maintenance of the stability of casein micelles. Since \( \kappa \)-casein is the only casein component containing sialic acid, the sialic acid content of casein mixture can be regarded as an index of the \( \kappa \)-casein content. Table I of Table I. Sialic Acid Content of Soluble Caseins  
\( \kappa \)-Casein content was calculated from the sialic acid content on the basis that the sialic acid content of the \( \kappa \)-casein was 2.2% as determined in this experiment. The sialic acid content of whole casein was 0.30%.  
The sialic acid content of the soluble casein formed by cooling was calculated from the amount of the soluble caseins and their sialic acid content before and after cooling.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sialic acid content of soluble casein (%)</th>
<th>( \kappa )-Casein content of soluble casein (%)</th>
<th>Amount of soluble casein (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uncooled</td>
<td>0.41</td>
<td>19</td>
<td>220</td>
</tr>
<tr>
<td>Heated</td>
<td>0.89</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>Uncooled</td>
<td>0.61</td>
<td>28</td>
<td>1330</td>
</tr>
<tr>
<td>Formed by cooling</td>
<td>0.52</td>
<td>24</td>
<td>1010</td>
</tr>
</tbody>
</table>

\( a \) The unheated and the heated concentrated WPF milk were cooled for 24 hr at 5°C.  
shows the sialic acid content of soluble caseins. \( \kappa \)-Casein content was calculated from the sialic acid content on the basis that the sialic acid content of \( \kappa \)-casein was 2.2% as determined in this experiment. In the case of the uncooled unheated concentrated WPF milk, the amount of soluble casein was so small that the sialic acid was not determined. The increment of the soluble casein by cooling was much larger in the heated concentrated WPF milk than in the unheated one as described previously. The sialic acid content of the soluble casein formed by cooling the heated concentrated WPF milk was calculated from the data before and after cooling. Evidently the sialic acid content of the soluble casein formed by cooling the heated concentrated WPF milk was larger than that of the soluble casein formed by cooling the unheated one.  
The determination of major casein components by DEAE-cellulose column chromatography was carried out in order to estimate a more exact composition of soluble caseins. The results are shown in Table II. The values of \( \kappa \)-casein content determined by DEAE-cellulose column chromatography were somewhat higher than those of \( \kappa \)-casein content estimated from the sialic acid content. This may be caused by heterogeneity in sialic acid content of \( \kappa \)-casein and the autolysis of casein during preparation of sample. When the period for the preparation of sample was prolonged, Fraction I and \( \kappa \)-casein fraction show an increase. The values of Fraction I in Table II were calculated from the amount of the soluble caseins and their composition before and after cooling.  
\( a \) Fraction I contains minor casein components such as TS- and \( \gamma \)-casein. \( b \) The unheated and the heated concentrated WPF milk were cooled for 24 hr at 5°C.

TABLE II. Composition of Soluble Caseins Determined by DEAE-CELLULOSE COLUMN CHROMATOGRAPHY  
The composition of whole casein was 57.4% of \( \alpha_\text{s} \)-casein, 22.8% of \( \beta \)-casein, 16.9% of \( \kappa \)-casein, and 2.9% of Fraction I.  
The composition of the soluble casein formed by cooling was calculated from the amount of the soluble caseins and their composition before and after cooling.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Composition of soluble casein</th>
<th>Amount of soluble casein (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \alpha_\text{s} )-Casein (%)</td>
<td>( \beta )-Casein (%)</td>
</tr>
<tr>
<td>Unheated</td>
<td>Uncooled 9.7</td>
<td>56.5</td>
</tr>
<tr>
<td></td>
<td>Cooled (^{a}) 21.8</td>
<td>25.0</td>
</tr>
<tr>
<td>Heated</td>
<td>Uncooled 20.7</td>
<td>37.7</td>
</tr>
<tr>
<td></td>
<td>Cooled (^{a}) 20.3</td>
<td>42.3</td>
</tr>
</tbody>
</table>

\( a \) Fraction I contains minor casein components such as TS- and \( \gamma \)-casein.
\( b \) The unheated and the heated concentrated WPF milk were cooled for 24 hr at 5°C.
became larger. Somewhat high content of Fraction I in soluble caseins seems to be caused by the autolysis of casein, because Kaminogawa et al.\textsuperscript{20,21} reported that TS- and \(\gamma\)-casein were possibly derived from \(\beta\)-casein by the hydrolysis with milk protease. The \(\alpha_s\) - and \(\kappa\)-casein contents of the soluble casein formed by cooling the heated concentrated WPF milk were larger than those of the soluble casein formed by cooling the unheated one, but \(\beta\)-casein content was reversed.

**DISCUSSION**

Casein micelles disaggregate ordinarily in the following two cases: (1) calcium or colloidal calcium phosphate is removed from casein micelles;\textsuperscript{22-24} (2) the interactions such as hydrogen or hydrophobic bond among casein components are disrupted.\textsuperscript{9,25} Heating concentrated milk above 105°C causes formation of soluble casein.\textsuperscript{3,4} Concentrating milk accelerates the formation of soluble casein by heating. In the previous paper,\textsuperscript{4} from the fact that the amount of soluble casein in the heated milk sample in which only casein micelles or ultrafiltrable fraction was augmented was small in comparison with that in the heated concentrated WPF milk, we assumed that the formation of soluble casein by heating might be attributed not only to the removal of calcium from casein micelles but also to the disruption of the interactions among casein components. Since the soluble casein formed by heating the concentrated WPF milk contains a large proportion of \(\kappa\)-casein, it seems probable that the formation of soluble casein participates in the aggregation of casein micelles by heating.\textsuperscript{5}

In the heated concentrated WPF milk, the amount of soluble casein increased remarkably during the first 1 hr of cooling and gradually thereafter, and the increment of soluble casein was much larger than that in the unheated one (Fig. 1). These facts suggest that the structure of casein micelles is loosened, though the aggregation of casein micelles occurs, by heat treatment of the concentrated WPF milk. According to Rose,\textsuperscript{6} colloidal calcium phosphate acts as a bonding agent within casein micelles and is a major factor controlling the distribution of micellar and soluble casein. When the effects of temperature and pH are combined by lowering the pH of cold milk, the increment of soluble casein is much greater than when either effect is measured singly. The partial solubilization of colloidal calcium phosphate, namely, the decrease of bonding action within casein micelles causes the marked increase of soluble casein by cooling. Since the amount of the soluble casein formed by cooling is much larger in the heated concentrated WPF milk than in the unheated one, the bonding action of colloidal calcium phosphate may be weakened by heating concentrated milk though the amount of colloidal calcium phosphate increases. We are carrying out further investigation on this problem.

The \(\kappa\)- and \(\alpha_s\)-casein contents of the soluble casein formed by cooling the heated concentrated WPF milk were higher than those of the soluble casein formed by cooling the unheated one (Tables I and II). In milk, \(\kappa\)-casein exists as a protective colloid and plays the most important role for the maintenance of the stability of casein micelles. It is well known that the soluble casein formed by cooling contains a large proportion of \(\beta\)-casein. According to Lin et al.,\textsuperscript{26} casein micelles are composed of the framework protein which is largely \(\alpha_s\)-casein and the dissociable protein which is mainly \(\beta\)- and \(\kappa\)-casein. Heating a concentrated milk system may cause looseness of the micellar framework.

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**REFERENCES**

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