SUSCEPTIBILITY OF κ-CASEIN COMPONENTS TO VARIOUS PROTEASES

Hiroshi Doi, Noboru Kawaguchi, Fumio Ibuki, and Masao Kanamori

Laboratory of Nutritional and Food Chemistry, Department of Agricultural Chemistry, Kyoto Prefectural University, Shimogamo, Kyoto 606, Japan
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Summary In order to clarify the function of the carbohydrate moiety of bovine κ-casein, κ-casein components having different carbohydrate contents were prepared by DEAE-cellulose chromatography. Five adsorbed fractions so obtained had an identical peptide chain and contained carbohydrate moieties of increasing size in the order of components P-2, P-3, P-4, P-5 and P-6. The susceptibility of κ-casein components, having different carbohydrate contents, to various proteases was examined. κ-Casein components were subjected to calf rennin [chymosin; EC 3.4.23.4], bovine trypsin [EC 3.4.21.4], α-chymotrypsin [EC 3.4.21.1], pronase [EC 3.4.24.4] and human plasmin [EC 3.4.21.7]. The component containing a larger carbohydrate moiety was less susceptible to hydrolysis than the component containing a smaller carbohydrate moiety. Rennin, trypsin, α-chymotrypsin and pronase hydrolyzed each component with a different reaction rate. On the contrary, human plasmin hydrolyzed component P-2, but did not hydrolyze component P-5. These results indicate that the carbohydrate moiety of κ-casein affected the susceptibility of κ-casein components to various proteases.

Keywords susceptibility, κ-casein, protease, casein, rennin, trypsin, α-chymotrypsin, plasmin, pronase, carbohydrate.

κ-Casein is important in preventing αs2- and β-casein from precipitating in the presence of calcium ion (1). When casein is attacked by rennin, κ-casein is hydrolyzed to para-κ-casein and macropепtide, resulting in the clotting of milk (2). κ-Casein is the specific substrate of rennin (3). This characteristic property is essential to cheese production and milk industry.

Casein is a phosphoprotein and the nitrogen source for mammalian infants, therefore, casein must be easily hydrolyzed by various proteases. κ-Casein is heterogeneous (4) and the function of the carbohydrate moiety is unclear.

1 土井裕司，川口 昇，伊吹文男，金森正雄
Treatment with proteolytic and glycolytic enzymes leads to the clotting or precipitation of κ-casein (5). The materials isolated from enzymic hydrolysates of κ-casein contain carbohydrates in different concentrations (6, 7).

We indicated in our previous paper that the heterogeneity of κ-casein was due to the carbohydrate moiety (8), therefore, the significance of the heterogeneity and the function of the carbohydrate moiety should be clarified. In this study, in order to achieve these purposes, various proteases were applied to κ-casein components having different carbohydrate contents and the difference in the susceptibility of the components was examined.

MATERIALS AND METHODS

Preparation of κ-casein components. κ-Casein components were prepared by the method described in the previous paper (8). κ-Casein was obtained from acid-precipitated whole casein by the method of ZITTLE and CUSTER (9) with slight modifications (8). κ-Casein was then chromatographed on a DEAE-cellulose column using a linear NaCl gradient (0.02–0.2 M) in 3 M urea/20 mM imidazole-HCl buffer, pH 7.0, containing 0.3% 2-mercaptoethanol. κ-Casein was composed of five adsorbed fractions which were the κ-casein components. They contained carbohydrate moieties of increasing size in the order of components P-2, P-3, P-4, P-5 and P-6.

Enzymes. Calf rennin was obtained from Nakarai Chemicals Co. (Lot No. M6H6280). Bovine trypsin (Type III) and bovine α-chymotrypsin (Type II) were purchased from Sigma Chemical Co. Pronase was from Kaken Chemical Co. (Kyoto, Japan). Human plasmin was a gift from Green Cross Co. (Osaka, Japan).

Enzyme assay methods. The rennin assay was carried out as described by MIYOSHI et al. (10). Hydrolys of κ-casein components by trypsin, α-chymotrypsin and pronase were carried out as described by CHIBA et al. (11). The human plasmin assay was performed under the same conditions, except for enzyme concentration, as described by EIGEL (12). The κ-casein component solution (20 mg/ml) was heated at 80°C for 10 min. The incubation mixture contained 20 mg of κ-casein component and 2.8 mg of plasmin in 2 ml of 0.05 M sodium tetraborate buffer, pH 8.4, and merthiolate (0.02%) to inhibit microbial growth. The reaction was stopped by the addition of an equal volume of 9 M urea containing 0.2% 2-mercaptoethanol.

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described in the previous paper (8).

Determination of amino groups. Amino group was determined by the method of SNYDER et al. (13) using trinitrobenzenesulfonic acid (TNBS), with the slight modification of adding 0.1% SDS to solubilize precipitates.

RESULTS

Treatment of κ-casein components with rennin

κ-Casein is the specific substrate of rennin. Rennin hydrolyzes κ-casein and
makes κ-casein lose its stabilizing effect for casein micelles. The coagulation of milk by rennin occurs by a primary proteolytic step followed by the aggregation of casein micelles. This characteristic reaction is utilized in the cheese-making industry. On the other hand, it was confirmed in our previous paper (8) that bovine κ-casein is heterogeneous due to the carbohydrate moiety. In this section, the rennin action on κ-casein components was examined by the method of MIYOSHI et al. (10). The results are shown in Fig. 1. Component P-2 contains no carbohydrate moiety and component P-6 contains the largest. Rennin acted on component P-2 releasing the largest amount of para-κ-casein; component P-6 was hardly hydrolyzed by rennin. All components have an identical peptide chain, but the carbohydrate content differs among components (8). Therefore, it is considered that the difference in rennin action among components is dependent on the carbohydrate content. SABARWAL et al. (14) reported the rennet susceptibility of acid and micellar casein in colostrum. Micellar casein gave a higher sialic acid release by rennet action, while the sialic acid content in micellar casein was lower than that in acid casein. These results indicate that the carbohydrate moiety lowered the susceptibility of κ-casein components to rennin.

The proteolysis of κ-casein components by trypsin

In order to examine the susceptibility of κ-casein components, many proteases with different specificities were used. Trypsin secreted from pancreas has a distinct specificity. In general, proteolytic activity is determined by the increase of trichloroacetic acid (TCA)-soluble materials. In this study, the action of trypsin on each component was followed by the increase of 3 and 12% TCA-soluble materials (Fig. 2). Trypsin hydrolyzed all components. In particular, the increase of acid-
Fig. 2. Increase of TCA-soluble material in κ-casein component by trypsin. The reaction was carried out in 40 mM Tris-HCl buffer, pH 7.2, at 25°C. The reaction mixture contained 0.75 mg of protein and 7.5 μg of trypsin in a volume of 1.5 ml. The reaction was stopped by adding 1.5 ml of 6 or 24% TCA. The reaction mixture was then allowed to stand at room temperature for about 30 min and the precipitate was removed by centrifugation. TCA-soluble material was measured by absorbance at 280 nm. A: Increase of 3% TCA-soluble material. B: Increase of 12% TCA-soluble material. ○—○, Component P-2; ●—●, component P-3; ▲—▲, component P-5; □—□, component P-6.

Fig. 3. Increase of amino groups by the reaction of κ-casein component with trypsin. The reaction was carried out in 10 mM Tris-HCl buffer, pH 7.0, at 25°C. The reaction mixture contained 2 mg protein and 20 μg trypsin in a volume of 0.5 ml. The reaction was stopped by adding 0.25 ml of 0.005 N HCl. In order to determine amino groups, 1.88 ml of 0.1 M sodium tetraborate buffer, pH 9.3, and 0.02 ml of 10% SDS were added to 0.1 ml of the reaction mixture, then 0.05 ml of 0.03 M TNBS was added. Increase of amino groups was determined by measurement of the difference of absorbance at 420 nm between the reaction mixture and control. ○—○, Component P-2; △—△, component P-4; □—□, component P-6.
soluble material in the case of component P-2 was the largest and this component was hydrolyzed to the greatest extent of all the components. However, measurement of the increase of TCA-soluble materials does not necessarily reflect the proteolytic activity. Therefore, in order to confirm the results mentioned above, the reactions were followed by the determination of amino groups using the TNBS method. Figure 3 shows the increase of amino groups produced in the hydrolysis of the $\kappa$-casein component by trypsin. Component P-6 was also hydrolyzed, but the increase of amino groups in the hydrolysate by trypsin was less than that in any other component. The results also indicate that component P-2, containing no carbohydrate, was hydrolyzed to the greatest extent.

The proteolyses of $\kappa$-casein components by $\alpha$-chymotrypsin and pronase

Trypsin cleaves the peptide bond in which the carboxyl group of lysine or arginine residue participates. On the other hand, $\alpha$-chymotrypsin is the proteolytic enzyme that cleaves the peptide bonds in which the carboxyl groups of tyrosine, phenylalanine residues etc. participate. Pronase is a mixture of several proteolytic enzymes and has a very wide specificity. The actions of $\alpha$-chymotrypsin and pronase on $\kappa$-casein components were examined under the same conditions as those of the proteolysis by trypsin. These results are shown in Figs. 4 and 5.

The increase of 3% TCA-soluble material was more remarkable in the case of the action of $\alpha$-chymotrypsin on component P-2 than on any other component. Using 12% TCA as the precipitating reagent, TCA-soluble material was produced.
by $\alpha$-chymotrypsin in the decreasing order of components P-2, P-4 and P-6. These results indicate that component P-2 was very easily hydrolyzed by $\alpha$-chymotrypsin and produced more TCA-soluble material than did components P-4 and P-6.

In addition, in the action of pronase on $\kappa$-casein components, component P-2 was more easily hydrolyzed than component P-3, and the latter was in turn more easily hydrolyzed than component P-4 (Fig. 5). At the higher TCA concentration, less TCA-soluble material of component P-4 was produced by pronase than component P-2 (Fig. 5B). These results indicate that component P-4, containing a large carbohydrate moiety, was less susceptible to hydrolysis by pronase than component P-2, containing no carbohydrate.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Action of $\kappa$-casein component with pronase. Enzyme assay was carried out as described in Fig. 2. A: Increase of 3\% TCA-soluble material. B: Increase of 12\% TCA-soluble material. O--O, Component P-2; ●●, component P-3; △△, component P-4.}
\end{figure}

The action of plasmin on $\kappa$-casein components

Rennin, trypsin and $\alpha$-chymotrypsin are proteolytic enzymes related to the digestion. Additionally, susceptibility to the plasma proteolytic enzyme, plasmin, was examined as well as the digestive proteolytic enzymes.

It is well known that protease exists in milk (15, 16). KAMINOGAWA et al. (17) reported that bovine milk protease was similar to plasmin. Bovine plasmin hydrolyzed $\beta$-casein and $\gamma_1$A$^2$, $\gamma_2$A$^2$ and $\gamma_3$A caseins were identified as being the products (12). However, the incubation of bovine plasmin with $\kappa$-casein A gave an unchanged disc gel electrophoretic pattern (18). In our experiment, human plasmin was used as the non-digestive proteolytic enzyme with $\kappa$-casein components.
fractionated on DEAE-cellulose as the substrate. The action of human plasmin on the $\kappa$-casein components was followed by SDS-polyacrylamide gel electrophoresis (Figs. 6 and 7). The activity of human plasmin was ascertained by the hydrolysis of

bovine $\alpha_{s1}$-casein. Component P-2, which contained no carbohydrate, was hydrolyzed and the fragment of component P-2 was detected in SDS-polyacrylamide gel electrophoresis. On the other hand, the SDS-polyacrylamide gel electrophoretic pattern showed no difference between the native component P-5 and the component P-5 treated with human plasmin. This result indicates that component P-5, which contains a large carbohydrate moiety, was not hydrolyzed. As native $\kappa$-casein gives a wide band, consisting of several bands due to its

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**Fig. 6.** SDS-polyacrylamide gel electrophoretic patterns of component P-2 after incubation with human plasmin. The reaction was performed as described in MATERIALS and METHODS. Electrophoresis was performed in pH 7.2–10.0% gel. Fifty $\mu$g protein was applied to each column.

**Fig. 7.** SDS-polyacrylamide gel electrophoretic patterns of component P-5 after incubation with human plasmin. The reaction and electrophoresis were performed under the same conditions as described in Fig. 6.
heterogeneity, on disc gel electrophoresis containing urea, it is not possible to detect the hydrolysis of \( \kappa \)-casein by using this method. Therefore, SDS-polyacrylamide gel electrophoresis is more suitable rather than disc gel electrophoresis to detect the hydrolysis of \( \kappa \)-casein. By treatment with human plasmin as well as proteolytic enzymes related to the digestion, the \( \kappa \)-casein component containing no carbohydrate moiety, component P-2, was hydrolyzed. Furthermore, it is worth noting that component P-5, containing a large carbohydrate moiety, was not hydrolyzed by human plasmin, but that this component was hydrolyzed by other proteases though the hydrolysis rate was very low. It is considered that this is due to the difference of digestive proteases from plasma protease.

**DISCUSSION**

The susceptibility of \( \kappa \)-casein components to various proteolytic enzymes was studied. \( \kappa \)-Casein is responsible for the stabilization of casein micelles. Therefore, this study is one step towards clarifying the function of \( \kappa \)-casein components, especially the carbohydrate moiety.

In general, it is considered that the carbohydrate moiety of glycoprotein does not contribute to its primary function (19). In the case of \( \kappa \)-casein, the carbohydrate moiety did not take part in the stabilization of casein micelles or the self-association (8). The carbohydrate moiety of \( \kappa \)-casein may relate to any secondary function in biological activity, for example, membrane permeation in secretion or the protection of plasma proteases. It is considered that the catabolism of asialoglycoprotein in mammals occurs in the peptide chain before the carbohydrate moiety (19). More recent work indicates that catabolism of some of the serum glycoproteins and hormones may be regulated through their carbohydrate moiety (20). Hence, the degradation of \( \kappa \)-casein components with different carbohydrate contents was studied *in vitro* with various proteases. In this study digestive enzymes, microbial protease and plasma protease were used.

These digestive enzymes acted on all \( \kappa \)-casein components though the reaction rate differed due to the differing carbohydrate contents. A component containing a larger carbohydrate moiety was less susceptible to hydrolysis than a component containing a smaller carbohydrate moiety. In the case of the action of trypsin and \( \alpha \)-chymotrypsin, it is confirmed by incubation with synthetic substrates that the difference in the reaction rate is not due to protease inhibitory activity. Microbial protease, pronase, hydrolyzed each component with a difference in reaction rate. On the contrary, human plasmin did not hydrolyze component P-5 that contains a large carbohydrate moiety (Fig. 7). It is unknown whether the discrepancy between our results and that of EIGEL (18) is due to the difference in the plasmin source, \( \kappa \)-casein employed or the method of activity measurement. In any treatment with various proteases, the carbohydrate content of a \( \kappa \)-casein component affected susceptibility to proteases.

SLATTERY and EVARD (21) reported that casein micelles consisted of subunits of variable composition and that the hydrophilic, carbohydrate-containing portion of
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\( \kappa \)-casein were near the surface. Therefore, one function of the carbohydrate moiety may be protection from plasma proteases. Further study is needed for clarification of the carbohydrate moiety function.

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