STUDIES ON κ-CASEIN

II. Effect of κ-casein heterogeneity on casein complex formation

Mohammed Yousef KHALIFA*, Ryoya NIKI and Shunrokuro ARIMA

Institute of Dairy Science, Faculty of Agriculture, Hokkaido University, Sapporo 060, JAPAN
*Permanent address: Dairy Science Department, Faculty of Agriculture, Tanta University, Kafr El-Sheikh, EGYPT

Received April 1, 1985

Introduction

It is known that $\alpha_{s'}$- and β-caseins form a stoichiometric complex with κ-casein and that the complex is stable against calcium ions while $\alpha_{s'}$- and β-caseins are precipitated by the ions. The formation of the stable complex is an important factor involved in the stabilization of casein micelles. κ-Casein is of prime importance for formation of the micelles and for maintaining their structural integrity. Although κ-casein is heterogeneous due to the differences in its sialic acid content, unfractionated preparation had used in the previous investigations related to the formation of casein complex. On the other hand, DOI *et al*[6] studied the complex formation of κ-casein components with $\alpha_{s'}$-casein and with β-casein. Carbohydrate moieties in proteins generally lead to better stability of the glycoproteins. However, the precise contribution of the the overall milk coagulation process is still controversial. Glycopeptides released from κ-casein contain 97 to 100% of the sialic acid originally attached to κ-casein[9] after rennet action and almost all of the sialic acid was released from the micelle by neuraminidase[17].

Covalently-bound carbohydrates (with their high concentration of hydroxyl groups) in the peptide would increase the hydrophilic properties and, therefore, the stability of the micelles. Several workers consider the carbohydrate as contributing significantly to the stability of the κ-casein monomer and thus to the stability of the micelle[4,6,23,26]. BAKRI and WOLFE[3] and MULLINS and WOLFE[18] claimed that the mere removal of carbohydrate form κ-casein was sufficient to cause coagulation of micelles. In contrast, the

carbohydrate moiety was considered to be neither necessary for stability of the micelle nor participating in any way in enzymic coagulation[1,9,16,17].

In this paper, compex formation of κ-casein fractions, having various sialic acid content, with a mixture of α_s- and β-casein in the absence and/or presence of $CaCl_2$ was investigated using gel filtration technique. The main aim of this study is to obtain additional information about the role of carbohydrate moiety, principally sialic acid, in the complex formation and stability.

Materials and Methods

Preparation of casein:

κ-Casein was prepared from bulk bovine milk by the urea-sulfuric acid method of ZITTLE and CUSTER[28] with some modification. Purification was

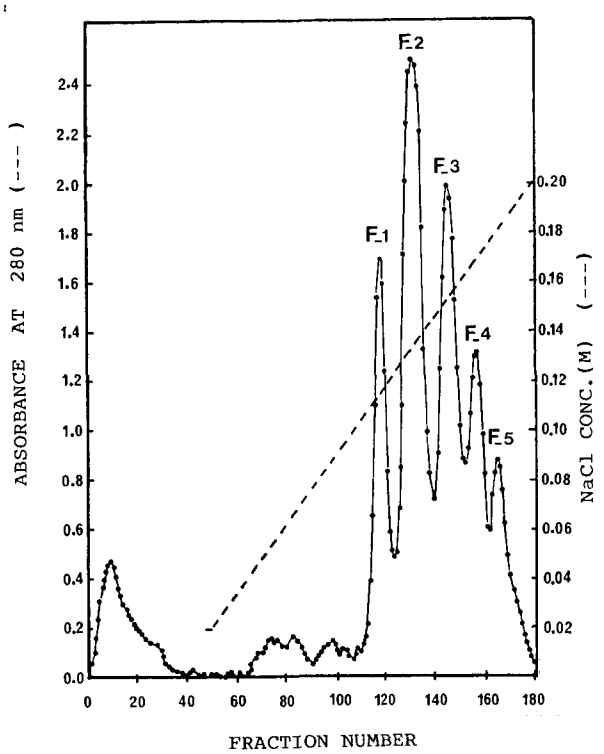

Fig. 1. DEAE-cellulose chromatographic pattern of κ-casein prepared by the modified urea-sulfuric acid method. 900 mg of κ-casein was dissolved in 45 ml of the starting buffer and reduced with 2-mercaptoethanol under nitrogen gas at 5°C for 16 hr. Reduced κ-casein was applied to a DEAE-cellulose column (4.5×20 cm). A flow rate was mainteined at 85 ml per hr. One tube containing 10 ml.

performed according to the method of NAKANISHI and ITOH[19].

α_s-Casein was prepared by modified urea procedures of ZITTLE *et al*[27] and purified according to the method of ZITTLE and CUSTER[28].

β-Casein was prepared by the method of ASCHAFFERNBURG[2] and purified according to the method described by NIKI *et al.*[20].

SH-reduced whole κ-casein was fractionated by Diethylaminoethyl (DEAE) cellulose column chromatography, using 3 M urea/20 mM imidazole-HCl buffer, pH 7.0 containing 0.3% mercaptoethanol, and a sodium chloride gradient eluent. Five adsorbed fractions, that differed in their sialic acid content, could obtained (Fig. 1). Sialic acid content of the fractions increased with sodium chloride concentration, in the gradient eluent, increasing. Fraction 1 is sialic acid free, F-2 is sialic acid-poor fraction and the other are sialic acid-rich ones. Polyacrylamide gel disc electrophoresis was carried out by the method of DAVIS[5] with some modification as reported in the previous paper[13].

Gel filtration:

Gel filtration chromatography was carried out at 37°C on a water-jacketed column (1.6×43.5 cm) of Toyopearl HW-65 (fractionation range is 5×10^4–5×10^6, equal to Sepharose 4B, 2B). A solution of 10 mM imidazole-HCl buffer, pH 7.1, containing 70 mM KCl and 0.01% sodium azide was used as a standard buffer. In the case of casein complex formation in the presence of calcium ions, solid calcium chloride was added to give calcium chloride concentration of 10 mM. No regard was paid for the slight changes in ionic strength. A mixtures of α_s- and β-caseins (5 mg of each) were dissolved in 1 ml 0.5% κ-casein fraction solution, afterwards 1 ml of the standard buffer or the standard containing 20 mM $CaCl_2$ was added after the casein mixture had completely dissolved (for 18 hrs at 5°C). The pH of the sample was adjusted to 7.1 and then the samples were held for one hour at 37°C in controlled water bath prior to gel filtration. The sample was chromatographed downwards at a constant flow rate of 25 ml/hr. Fractions of 2 ml were collected, and their UV absorbances were measured at 280 nm using a Hitachi Spectrophotometer model 220-A (Hitachi Ltd., Japan) with a flow cell (light path 5 mm).

Gel column calibration:

Blue dextran 2000 was used for cheking column packing irregularities and column void volume (V_0) determination.

The calibration kit proteins were dissolved in proper combination in the eluent buffer. Toyoperal HW-65 column was calibrated with the following

proteins : rabbit muscle aldolase (MW=158,000) ; bovine liver catalase (MW= 232,000) ; horse spleen ferritin (MW=440,000) and bovine thyroid thyroglobulin (MW=669,000), all obtained from Pharmacia Fine Chemicals, Sweden.

Results and Discussion

In the absence of $CaCl_2$:

κ-Casein fractions were mixed with a mixture of α_s- and β-casein in the absence of calcium ions. The aim of this experiments is to throw some light on the properties of casein complex and the effect of sialic acid content on these properties. A 2 ml sample of 0.75% protein solution (α_s-, β- and κ-caseins, each 2.5 mg/ml) was applied on the gel column. Using the standard imidazole buffer, as an eluant, the sample was chromatographed downwards. The gel filtration patterns of complexes formed in the absence of calcium ion are illustrated in Fig. 2. This figure indicates that κ-casein and each κ-casein fraction were able to form complexes with a mixture of α_s- and β-casein at 37°C.

The elution chromatograms showed that only two peaks were obtained. A minor peak, that eluted in the fornt, was followed by the major one. In all elution profiles the minor peak was observed with a maximum elution at elution volume of 28 ml, that is equal to the column void volume. On the other hand, the major peak in all κ-casein fractions was well-defined and had the same position. It was eluted with a maximum at elution volume of 44 ml. In the case of the complex that formed with unfractionated κ-casein, it had a major peak with elution volume of 42 ml.

The manner of complex formation observed somewhat resembled to that between α_{s_1}- and κ-casein[6,15,21,22,24]. However, the magnitude of the interaction was variable because different experimental conditions were used. The magnitude of the κ- : α_s- : β-caseins complex formation is influenced by both of the concentration of protein in the samples (7.5 mg/ml) and high temperature of gel filtration column which should encourage the association.

Using calibration technique (Fig. 3), all κ-casein fractions could form complexes, that appeared in the slow-eluted peak, with molecular weights of about 6.6×10^5 while the complex formed with unfractionated κ-casein had molecular weight of about 8.7×10^5.

These results are in good accordance to the results of KAMINOGAWA *et al*[11]. They obtained the value of 4.5×10^5 for the molecular weight of the α_{s_1}-κ-casein complex occurring in a neutral solution free of calcium. Similar results were obtained by DOI *et al*[6] in their studies on the interaction of κ-casein components with α_{s_1}- and β-casein. They reported that the

molecular weight of complexes of unfractionated κ-casein with both α_{s_1}-casein and β-casein were about 7×10^5, in the absence of $CaCl_2$, while those of complexes of each κ-casein component with α_{s_1}- and β-casein were about 5×10^5. The values obtained through this study were a little higher because of using a mixture of α_s- and β-casein with κ-casein fractions.

The chromatographic diagram of fraction 5 had the smallest elution

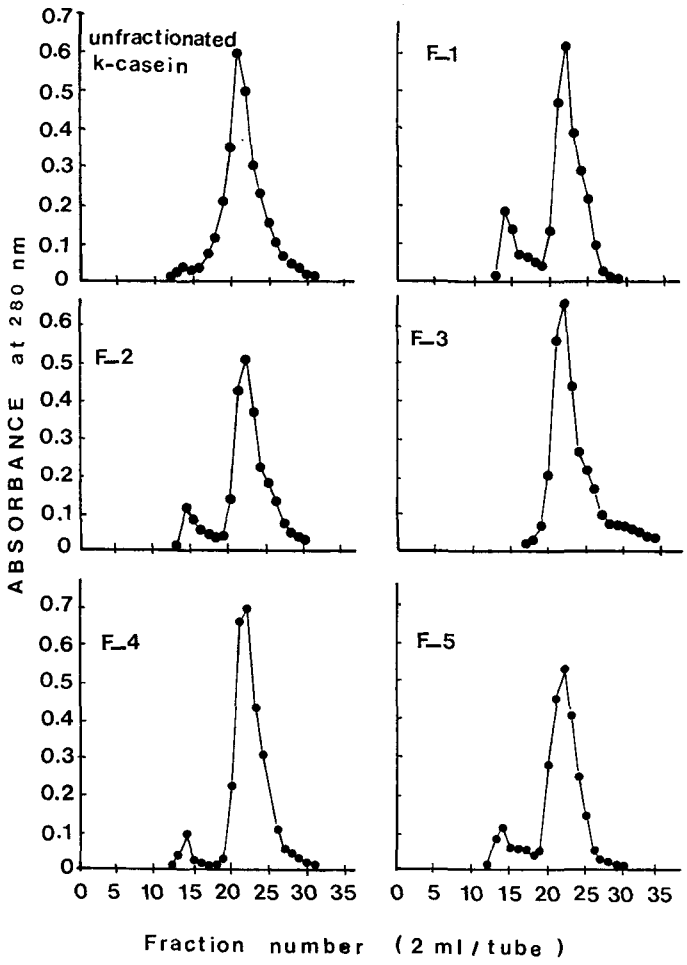

Fig. 2. Gel filtration patterns of κ-casein fractions with a mixture of α_s- and β-casein (5 mg each) in the absence of calcium. Two ml of the sample solution were applied on Toyopearl HW-65 column (1.6×43.5 cm) at 37°C using 10 mM imidazole-HCl buffer (pH 7.1) containing 70 mM KCl and 0.01% NaN_3. The flow rate was maintained at 25 ml/h and 2 ml fractions were collected.

volume (Fig. 2). The complex formed with unfractionated κ-casein had an elution pattern with faster elution rate than all other complexes formed with κ-casein fractions. Moreover, its elution was accompanied by a slight tailing compared with that of complexes with fractions 1 and 5. On the other hand, the complex formed with κ-casein fraction 3 had a pronounced skewed tailing edge with the smallest minor fast-eluted peak that appeared at the column void volume. The electrophoretic patterns of eluted peaks show that the majority of all components are presented in the major slow-eluted peak. On the other hand, the fast-eluted portion contained only traces of casein components (Fig. 4 A).

It is possible to conclude that, in the absence of calcium ion, the gel filtration profiles of the complexes of κ-casein fractions, having sialic acid, were very similar to that of sialic acid-free fraction. Moreover, sialic acid

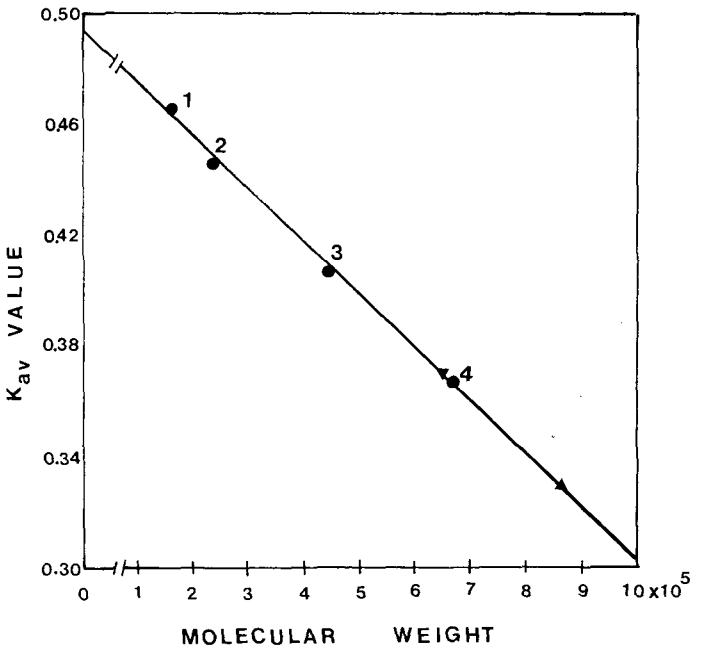

Fig. 3. Calibration curve of Toyopearl HW-65 column with proteins of known molecular weight.

1-Rabbit muscle aldolase (158,000).
2-Bovine liver catalase (232,000).
3-Horse spleen ferritin (440,000).
4-Bovine thyroid thyroglobulin (669,000).
The solvent in all cases was the standard buffer.
◀ Complex with MW of 6.6×10^5, ▶ Complex with MW of 8.7×10^5.

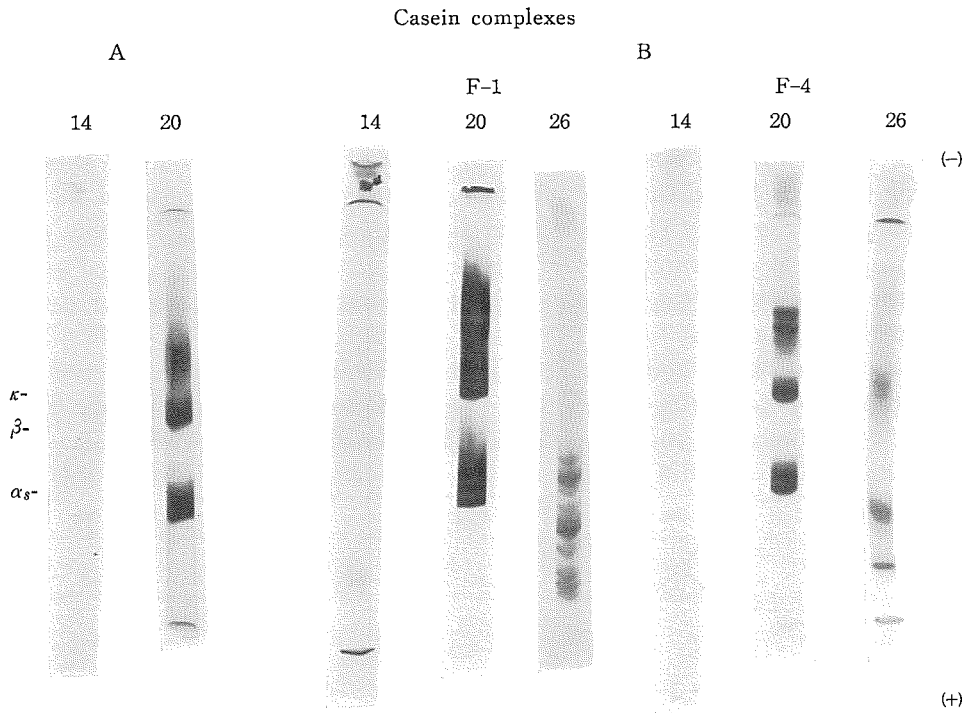

Fig. 4. Polyacrylamide gel disc electrophoretic patterns of casein complexes formed by mixing κ-casein fractions with a mixture of α_{s}- and β-casein.
A : In the absence of calcium.
B : In the presence of 10 mM $CaCl_2$.
Numbers express the fraction number of the gel filtration. Electrophoresis was carried out in trisglycine buffer (pH 8.6) at 3 mA per gel for 1.5 hr. Migration was from up (cathode) to down (anode).

content had no role in casein complex formation. On the other hand, the pattern of unfractionated κ-casein complex was similar to those of κ-casein fractions except that the second peak was eluted faster with a high molecular weight relative to those of the fractions. Similar results had been obtained by DOI *et al*[6]. They found that complexes of κ-casein components having carbohydrate (P-3 to P-6) not only with α_{s_1}-casein but also with β-casein were very similar to each other. Also they reported that complex of carbohydrate-free component (P2) with α_{s_1}- or β-casein was eluted as a single peak with a tailing edge. Similar findings had also been obtained by PEPPER[22] using a carbohydrate-free κ-casein. He obtained a single peak with a skewed tailing using a ratio of 1 : 1 (α_{s_1}- : κ-casein). KAMINOGAWA *et al.*[12] used unfractionated κ-casein but it gave a complex with a single broad peak with α_{s_1}-

casein. Moreover, KUDO and MADA[15] could obtain a complex of α_{s_1}-casein and one carbohydrate moiety κ-casein with two peaks in the absence of $CaCl_2$;

From Fig. 2 it is possible to observe that the complexes formed with κ-casein fractions and a mixture of α_s- and β-casein are stable. Complex formation between κ-, α_s- and β-casein does not occur in the absence of Ca^{2+} at the same conditions as in its presence[10]. The association between casein components includes mainly hydrophobic interactions and possibly hydrogen bonds[7,8]. Moreover, it is possible that hydrophobic interaction may play a role in certain stage of complex formation and may facilitate subsequent interactions between casein components around cysteine residues. Also, electrostatic association could have been involved in the complex formation.

In the presence of $CaCl_2$:

Of primary interest to milk protein chemistry is the binding of calcium to the caseins because of its involvement in micelle formation and its effect upon the stability of the milk protein system during processing. The addition of calcium favored aggregation in micelles. Both α_s- and β-caseins have regions in their molecules that possess high concentrations of polar groups which result in strong interactions. Calcium may form both inter- and intramolecular bridges in the caseins[25].

The ability of κ-casein fractions, that differ in their sialic acid content, to form complexes with a mixture of α_s- and β-casein in the presence of $CaCl_2$ was investigated. Sample (2 ml) of 0.75% casein mixture, with a weight ratio of 1 : 1 : 1 (κ-, α_s- and β-caseins), was applied to the gel chromatographic column at 37°C. The imidazole-HCl standard buffer, containing 10 mM $CaCl_2$ was used as an elution buffer. The collected 2 ml eluates were subjected to UV absorbance measurements at 280 nm.

Results in Fig. 5 show that the elution profiles consist of a series of peaks or multicomponent interaction. The fast-eluted peak was eluted in the position of the column void volume and had an area that decreased proportionally with the increasing sialic acid content. This portion may be due to the polymerization of casein components that occurred together through hydrophobic and electrostatic bonds. It contains only traces of polymerized casein components.

The major slow-eluted peaks are symmetrical and eluted with a maximum at elution volume of 40 ml in the complexes formed with unfractionated κ-casein as well as with all κ-casein fractions except that formed with Fraction 4, which had a peak at elution volume of 42 ml. The major peak of unfractionated κ-casein was eluted faster than those of all κ-fractions with

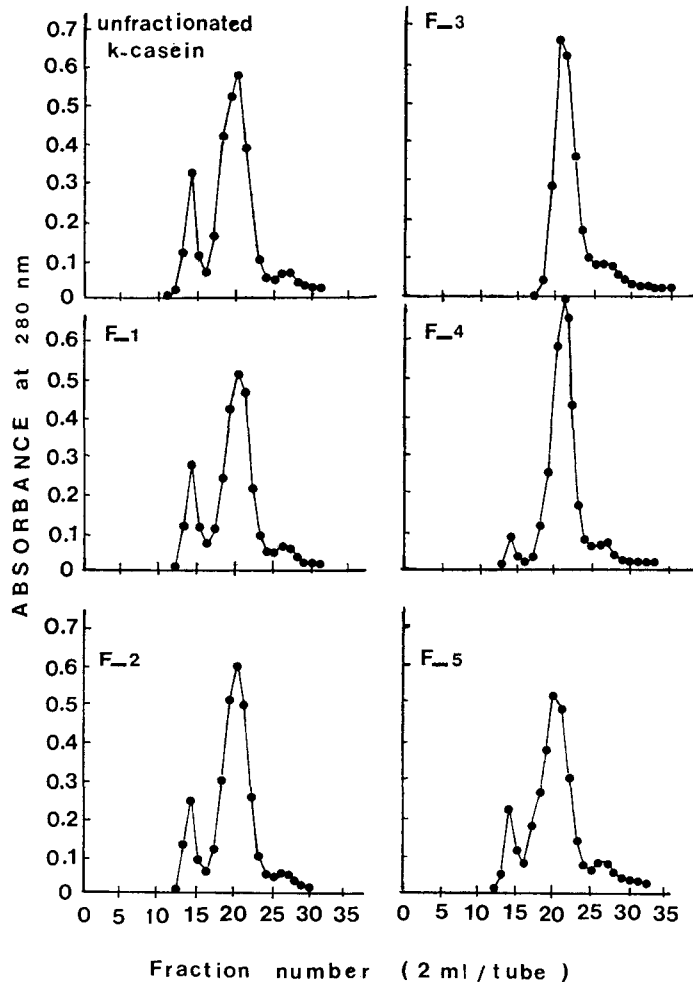

Fig. 5. Gel filtration patterns of κ-casein fractions with a mixture of α_s- and β-casein (5 mg each) in the presence of calcium. Two ml of the sample solution were applied on Toyopearl HW-65 column (1.6×43.5 cm) at 37°C using 10 mM imidazole-HCl buffer (pH 7.1) containing 70 mM KCl, 0.01% NaN_3 and 10 mM $CaCl_2$. The flow rate was maintained at 25 ml/h and 2 ml fractions were collected.

a skewed tailing edge. On the other hand, fraction 3 formed a complex with a slower elution relative to Fractions 1, 2 and 5 and had a discrete skewed tailing end. Fraction 4, that contains much sialic acid, formed a complex with the slowest elution rate of all fractions. The major peak contains all casein fractions in approximetely equal content (Fig. 4 B).

The third peak an additional minor one that eluted later at the elution volume of 52–54 ml. The area under this minor peak was approximately equal in all fractions. This portion may be resulted from limited hydrolysis of casein components during their preparation and purification, resulting in small molecules that migrated faster in PAG-electrophoresis.

The above results are consistent with those of KUDO and MADA[15]. They could obtain a complex of α_{s_1}-, β- and κ-caseins eluted through series of two peaks in the presence of 15 mM $CaCl_2$. Although pronounced shifts of peak areas were observed, the elution profiles of the complex formed from mixing α_{s_1}- and κ-casein showed three peaks as those observed in this work.

By the column calibration technique (Fig. 3), the majority of the formed complexes had a molecular weight of about 1.1×10^6 for all complexes except that formed with Fraction 4, which had the smallest molecular weight of about 8.7×10^5. The components that were present in the minor very slow-eluted peak had a molecular weight less than 1×10^5. It may be considered to be a monomer or dimer of casein components, mainly α_s- and β-caseins.

From the above results it is possible to conclude that each κ-casein fraction could form complex with a mixture of α_s- and β-casein in the presence of calcium chloride. The presence of $CaCl_2$ facilitated the association of casein components into large polymers. It may be assumed that the presence of carbohydrate, sialic acid, plays a role in the complex formation in the presence of $CaCl_2$. The sialic acid-rich fraction gave a complex with lower molecular weight compared with sialic acid-free fraction. The high concentration of hydroxyl groups of the covalently-bound carbohydrate on the protein chain will increase the hydrophilic properties. This will lead to form a stable and smaller molecular weight complex. On the other hand, it can be noticed that the molecular weight of the complexes formed in the presence of $CaCl_2$ was higher than that of the complexes formed in the absence of $CaCl_2$. This may be the results of the association of casein components that is mediated by calcium ions via the interaction between Ca^{2+} and phosphate residues of α_s- and β-casein molecules.

Investigations using stains specific for glycosylated κ-casein indicated that the glycosylated κ-casein is located mainly in the outer layer of large micelles, whereas for small micelles it is distributed evenly throughout[14]. Seventy five percent of micellar sialic acid is on the micelle surface[17]. Also it is reported by MACKINLAY and WAKE[16] that aggregation or interaction of κ-casein with other casein components may prevent addition of carbohydrate to all molecules.

The above results are consistent with those of DOI *et al*[6] that all κ-casein fractions could form complexes with both of α_{s_1}- and β-caseins. In contrast, they reportred that the complexes formed by κ-casein components gave very similar patterns to each other. This may be due to the differences of this work and their work conditions. Also, similar results were obtained with a mixture of α_{s_1}- or α_{s_2}- and κ-casein in the presence of 7 mM $CaCl_2$ by KUDO and MADA[15].

Summary

κ-Casein was fractionated on diethylaminoethyl cellulose column into five adsorbed fractions with various sialic acid content. κ-Casein fractions were mixed with a mixture of α_s- and β-casein, in the weight ratio of 1 : 1 : 1. The interactions were examined at 37°C by using Toyoperal HW-65 (concur to the properties of Sepharose 4 B and 2 B) gel chromatography. Each κ-casein fraction had the ability to form complexes with α_s- and β-casein mixture either in the absence or presence of 10 mM $CaCl_2$. Gel filtration patterns of all κ-casein fractions in the absence of $CaCl_2$ were very similar with molecular weight of about 6.6×10^5. However, unfractionated κ-casein formed a complex with molecular weight of about 8.7×10^5. In the presence of $CaCl_2$, the fractions gave larger complexes compared with those in the absence of $CaCl_2$. Moreover, the sialic acid-rich fraction formed a complex with molecular weight smaller than that of complex formed with sialic acid-free fraction. Moelcular weights of the complexes formed in the presence of $CaCl_2$ were 8.7×10^5 and 1.1×10^5 for sialic acid-rich and sialic acid-poor fractions, respectively. The heterogeneity of κ-casein leads to form complexes with different molecular weight and that the sialic acid contributes to the stability of casein complex.

Literature Cited

1. ARMSTRONG, J. McD., MCKENZIE, H. A. and SAWYER, W. H.: "On the fractionation of β-lactoglobulin and α-lactalbumin." *Biochim. Biophys. Acta*, **147**: 60–72. 1967
2. ASCHAFFENBURG, R.: "Properties of β-casein by a modified urea fractionation method." *J. Dairy Res.*, **30**: 259–260. 1963
3. BAKRI, M. and WOLFE, F. H.: "Destabilization of casein micelles by lysozyme." *Can. J. Biochem.*, **49**: 882. 1971
4. BEEBY, R. and NITSCHMANN, H.: "The action of rennin on casein. The disruption of the κ-casein complex." *J. Dairy Res.*, **30**: 7–16. 1963
5. DAVIS, B. J.: "Disc electrophoresis. II. Methods and application to human serum proteins." *Ann. N. Y. Acad. Sci.*, **121**: 404–427. 1964

6. DOI, H., IBUKI, F. and KANAMORI, M.: "Interactions of κ-casein components with α_{s_1}- and β-caseins." *Agric. Biol. Chem.*, **43**: 1301–1308. 1979
7. DOSAKO, S., KAMINOGAWA, S. and YAMAUCHI, K.: "Thermodynamics of the interaction between α_{s_1}- and κ-caseins." *Agric. Biol. Chem.*, **39**: 2347–2351. 1975
8. DOSAKO, S., KAWAMURA, S., IZUTSU, T., HONDA, Y., FUKUSHIMA, M., KIMURA, T., MURATA, H. and TANEYA, S.: "Changes in rheological and chemical properties of casein dope solution through increase of aging time." *Agric. Biol. Chem.*, **43**: 803–807. 1979
9. GIBBON, R. A. and CHEESEMANN, G. C.: "Action of rennin on casein: the function of the neuraminic acid residues." *Biochim. Biophys. Acta*, **56**: 354–356. 1962
10. GREEN, M. L. "The specificity for κ-casein as the stabilizer of α_s-casein and β-casein. I. Replacement of κ-casein by other proteins." *J. Dairy Res.*, **38**: 9–23. 1971
11. KAMINOGAWA, S., DOSAKO, S. and YAMAUCHI, K.: "Studies on molecular properties of α_{s_1}-κ-casein complex by the hydrodynamic methods." *Agric. Biol. Chem.*, **38**: 2337–2341. 1974
12. KAMINOGAWA, S., KOIDE, K. and YAMAUCHI, K.: "Interaction of α_{s_1}-κ-casein complex with alkaline-earth-metal ions." *Agric. Biol. Chem.*, **41**: 697–702. 1977
13. KHALIFA, M. Y., NIKI, R. and ARIMA, S. "Studies on κ-casein. I. Fractionation and some properties of κ-casein fractions." *J. Fac. Agri., Hokkaido Univ.* (in press). 1985
14. KUDO, S., IWATA, S. and MADA, M. "An electron microscopic study of the location of κ-casein in micelles by periodic acid-silver methenamine staining." *J. Dairy Sci.*, **62**: 916–920. 1979
15. KUDO, S. and MADA, M.: "Interaction between α_{s_2}-casein and κ-casein as determined by gel filtration." *NZ J. Dairy Sci. Tech.*, **18**: 225–232. 1983
16. MACKINLAY, A. G. and WAKE, R. G. "Fractionation of S-carboxymethyl-κ-casein and characterization of the components" *Biochim. Bioyhys. Acta*, **104**: 167–180. 1965
17. MEHAIA, M. A. and CHERYAN, M.: "Treatment of casein micelles with soluble and immobilized neuraminidase: Implications in structure of the micelle." *J. Dairy Sci.*, **66**: 390–395. 1983
18. MULLINS, W. J. and WOLFE, F. H.: "Disc gel electrophoresis of caseins treated with proteolytic and glycolytic enzymes." *J. Dairy Sci.*, **57**: 9–14. 1974
19. NAKANISHI, T. and ITOH, T.: "Studies on the changes of the milk casein by various treatments. IX. On the preparation method of κ-casein by ZITTLE & CUSTER's method and some properties of κ-casein prepared by this method." *J. Agric. Chem. Soc. Japan*, **43**: 866–869. 1969
20. NIKI, R., ARIMA, S. and HASHIMOTO, B.: "Studies on the properties of casein fractionated on DEAE-cellulose." *Jap. J. Zootech. Sci.*, **34**: 47–56. 1963
21. NOBLE, R. W. Jr. and WAUGH, D. F.: "Casein micelles. Formation and structure." *J. Am. Chem. Soc.*, **87**: 2246–2257. 1965
22. PEPPER, L.: "Casein interactions as studied by gel chromatography and ultracentrifugation." *Biochim. Biophys. Acta*, **279**: 147–154. 1972

23. SINKINSON, G. and WHEELOCK, J. V.: "Carbohydrates of the glycopeptides released by the action of rennin on whole milk." *Biochim. Biophys. Acta*, **215**: 517-521. 1970
24. VOORT, F. R. van de, MA, C.-Y. and NAKAI, S.: "Molecular weight distribution of interacting proteins calculated by multiple regression analysis from sedimentation equilibrium data and interpretation of α_{s_1}-κ-casein interaction." *Archives of Biochem. Biophys.*, **195**: 596-606. 1979
25. WAUGH, D. F., CREAMER, L. K., SLATTERY, C. W. and DRESDNER, G. W.: "Core polymers of casein micelles." *Biochemistry*, **9**: 786-795. 1971
26. WHEELOCK, J. V. and KNIGHT, D. J.: "The action of rennet on whole milk." *J. Dairy Res.*, **36**: 183-190. 1969
27. ZITTLE, C. A., CERBULIS, J., PEPPER, L. and DELLAMONICA, E. S.: "Preparation of calcium sensitive α-casein." *J. Dairy Sci.*, **42**: 1897-1902. 1959
28. ZITTLE, C. A. and CUSTER, J. H.: "Purification and some of the properties of α_s-casein and κ-casein." *J. Dairy Sci.*, **46**: 1183-1188. 1963