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STUDIES ON κ -CASEIN

I. Fractionation and some properties of κ -casein fractions

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Introduction

κ -Casein is a secretory glycoprotein synthesized by the mammary gland and it is a part of the casein complex which constitutes about 80% of the protein cow's milk. κ -Casein accounts for approximately 15% of whole bovine casein.

κ -Casein appears to exhibit chemical heterogeneity as well as heterogeneity with respect to size. The chemical heterogeneity of cow κ -casein from pooled milk can be attributed to the genetic variants and also to the non-identical composition of the carbohydrate groups present. Indeed κ -casein from normal milk can be resolved by chromatography on DEAE-cellulose into sugar-free and sugar-rich fractions possessing the same peptide skeleton²⁵.

The carbohydrate part of κ -casein is a prosthetic group consisting of galactose, galactosamine and sialic acid combined approximately in the ratio of 1:1:1 and that this group may be linked to a polypeptide chain through threonine or serine^{17,19}.

It is now recognized that carbohydrates attached to proteins can contribute to, or modify, the properties and functions of the proteins¹⁶. κ -Casein contains substantial amounts of carbohydrates, which has been the subject of interest in recent years.

Since the original discovery of κ -casein⁵² several investigators have examined the electrophoretic and chromatographic properties of the isolated κ -casein. The κ -casein family consists of major carbohydrate-free component

and a minimum of six minor components^{1,2,8,9,18,21,24,25,33,37,38,40,49,60,61}. Structure of the minor κ -casein components have not been established, and considerable disagreement exists among investigators. Generally, the minor κ -casein components are believed to be glycosylated forms of the major κ -casein, but even this has not been confirmed by all investigators.

The stabilizing ability of κ -casein is very complicated. Many researchers studied the factors that affect it^{23,28-31,47,59,63,64,66}. However, the effect of carbohydrate on this property has not been studied in details.

One of the main purposes of the present work concerns with the role of the carbohydrate in κ -casein. Because κ -casein contains most of the carbohydrate of whole casein, and because of its unique role as a micelle stabilizer and substrate for rennin, this enzyme releases the carbohydrate portion of the molecule. It is natural to look for the involvement of the carbohydrate moiety in some of these functions. The heterogeneity of SH-reduced κ -casein was studied by DEAE-cellulose column chromatography. Partial characterization of each fraction has been examined.

Materials and Methods

Bulk bovine milk was obtained from Hokkaido University Dairy herd for all preparations of caseins. All the chemicals used in this work were analytical grade reagents.

Preparation of κ -casein :

κ -Casein was prepared from acid whole casein by the method described by ZITTLE and CUSTER⁶⁷ with some modifications. The amount of sulfuric acid was decreased to be 150 ml. κ -Casein in the filtrate was precipitated by the addition of 0.8 M ammonium sulfate instead of 1 M.

κ -Casein was purified by ethanol precipitation using the method of MCKENZIE and WAKE²⁷ with the modification of NAKANISHI and ITOH³². Purification was repeated twice to get highly purified fraction.

Preparation of α_s -casein :

α_s -Casein was prepared by modified urea procedures of ZITTLE *et al*⁶⁵ and purified according to the method of ZITTLE and CUSTER⁶⁷.

Fractionation of κ -casein by DEAE-cellulose chromatography :

900 mg of whole purified κ -casein were dissolved in 45 ml of the starting buffer and reduced with 2-mercaptoethanol under nitrogen gas at 5°C for 16 hrs. The reduced κ -casein was applied to the DEAE-cellulose column using linear NaCl gradient (0.02 to 0.2 M) in the starting buffer (3 M urea/

20 mM imidazole-HCl buffer, pH 7.0, containing 0.3% 2-mercaptoethanol). The fractions were collected by volumetric siphon attached with the fraction collector and examined by a Hitachi Spectrophotometer model 220-A (Hitachi Ltd., Japan) at 280 nm. Fractionated κ -casein components were dialyzed against deionized water containing 0.1% 2-mercaptoethanol at pH 7.0 for 48 hrs at 5°C, and then lyophilized.

Polyacrylamide gel disc electrophoresis :

Disc gel electrophoresis was carried out by modifications of the method of DAVIS⁷⁾ with an apparatus produced by Mitsumi Scientific Industry Co., Ltd., Japan. One volume of κ -casein fraction was mixed with an equal volume of 9 M urea solution, containing 1 M sucrose, followed by addition of 2-mercaptoethanol in small test tube. After sealing the tube, the contents were mixed well for 16 hrs. at 5°C. Samples (about 200 μ g of protein) were applied to 7.5% polyacrylamide gel containing 4.5 M urea, using a 0.5 \times 7.0 cm gel column and Tris-glycine buffer, pH 8.6. Small pore size gel was performed by mixing 3 ml of solution A⁷⁾, 6 ml of solution C⁷⁾, 15 ml of 7.2 M urea and 0.0168 gram of ammonium persulfate. This mixture was suitable for 12 gel columns.

Nitrogen determinations :

Total N was determined by the AOAC³⁾ procedures using the micro-Kjeldahl method.

Phosphorus determinations :

Total P was analysed by the colorimetric method of CHEN *et al.*⁵⁾ with some modifications. One ml of 0.5% κ -casein was ashed in the presence of concentrated sulfuric acid and nitric acid. The digest was transferred quantitatively with several washings of deionized water to a total volume of 10 ml in a volumetric flask. 4 ml of the diluted or the standard solution were mixed with 4 ml of reagent C (1 vol of 6 N H₂SO₄+2 vol of deionized water+1 vol of 2.5% ammonium molybdate+1 vol of 10% ascorbic acid, mixed well and prepared fresh each day) and placed in a 37°C water bath for 2 hrs. The optical density of the solutions was estimated by using Hitachi 220-A Spectrophotometer at 280 nm against the blank. A stock solution containing 1 mg P per ml was prepared by dissolving 1.0967 g KH₂PO₄ (dried in an air oven) in deionized distilled water and diluted to 250 ml. Suitable standard solutions for calibration of the spectrophotometer were obtained by dilution of the stock solution.

Determination of sialic acid :

Total sialic acid content determination was carried out according to the

thiobarbituric acid assay method of WARREN⁵⁰ with the modification of KIM *et al.*²². The samples were hydrolysed with 0.1 N H₂SO₄ for 1 hr. Sialic acid was identified by Hitachi 220-A Spectrophotometer at 549 nm. A standard curve was prepared with N-acetylneuraminic acid (Sigma Chemical Company) which was used as a standard for expressing the sialic acid content of κ -casein fractions.

Stabilization of α_s -casein by κ -casein :

The stabilizing ability of κ -casein fractions was measured according to the method of ZITTLE⁶⁶ with some modifications. The test was set up in 10-ml test tube, total volume of the test mixture was 5 ml. An 1% solution of the α_s -casein (7.5 mg), at pH 7.8 was added in the tubes, followed by the required volumes of water and amounts of 0.5% κ -casein fraction such that the κ -/ α_s -ratio varied from 0.05 to 0.20. Finally, 1 ml of 0.1 M calcium chloride was added and the mixture stirred with a spatula. The test mixtures were kept at 30°C in water bath for 30 min, then centrifuged at 3000×g for 5 min at room temperature. Samples of the supernatant solutions were withdrawn, 1 ml was diluted by 4 ml water and 2 drops of 0.05 N NaOH was added to clarify the dilutions. The casein in solution was determined from the light absorption at 280 nm using Hitachi 220 A Spectrophotometer.

Gel filtration :

Toyoperal HW-75 (fractionation range is $5 \times 10^5 - 5 \times 10^7$, equal to Sepharose 2B<) was used. One ml of 0.5% κ -casein fraction was applied directly to the flat, drained bed surface of the gel column and then chromatographed with the starting buffer (10 mM imidazole-HCl buffer, pH 7.1, containing 70 mM KCl and 0.01% sodium azide). Fractions of 2 ml were collected, using fraction collector model SF-100 G (Tokyo Kagaku Sangyo Co. Ltd., Japan) and their UV absorbances were measured at 280 nm using Spectrophotometer 220-A with a flow cell (light path 5 mm) and recorded by Hitachi recorder.

Gel column calibration :

Bule dextran 2000 was used for checking column packing irregularities and column void volume (V_0) determination. Blue dextran was fresh prepared (1.0 mg/ml) in the eluant buffer and its sample volume was 1% of the total gel bed volume (V_t).

The calibration kit proteins were dissolved in proper combination in the eluent buffer. The concentration of each protein was 5 mg/ml except ferritin which was 1 mg/ml and the sample size was 1% of the column V_t . Toyoperal HW-75 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. Calibration curve of Toyoperal HW-75 column with these proteins is shown in Fig. 5.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_e = elution volume for the protein.

V_0 = column void volume.

V_t = total bed volume,

Results

Heterogeneity of κ -casein :

Purified isoelectric κ -casein, reduced by 2-mercaptoethanol, was applied to DEAE-cellulose column and eluted at room temperature. About 50 fractions, each of 10 ml, were eluted with the starting buffer, the first eluent. The second eluent, the starting buffer containing a gradual increase in concentration of NaCl (0.02–0.20 M), was used until about 200 fractions were obtained. The absorbance values of the fractions were measured at 280 nm. A typical chromatogram (Fig. 1) could be obtained with the SH-reduced κ -casein on the DEAE-cellulose column. Presence of sharp explicit six peaks numbered 0 to 5 and a few minor ones (Figure 1) can be observed. The chromatogram peaks were separately examined by the polyacrylamide gel electrophoresis and the results are shown in Fig. 2.

The 10-ml fractions of eluate under each peak were separately combined somewhat arbitrarily, and the fractions numbered from 1 to 5 then packed in seamless cellulose tubing and were dialyzed. However, peak 0, which is eluted in the front with the starting buffer, was not adsorbed to the column and did not show any band on the PAG-electrophoresis. This peak is a trace of material and it may be assumed to have arisen from limited proteolysis during its preparation. Also, the minor peaks that eluted with the low concentration of NaCl did not give any band on the gel electrophoresis. It contained only traces of slow moving impurities present in the original preparation of κ -casein. The peak 0 and the other minor peaks accounted about 13% of the preparation (Table 1) and have not been studied further.

The dialyzed fractions were separately subjected to freeze-drying and then the fractions were stored under frozen condition till analyse. The yield of

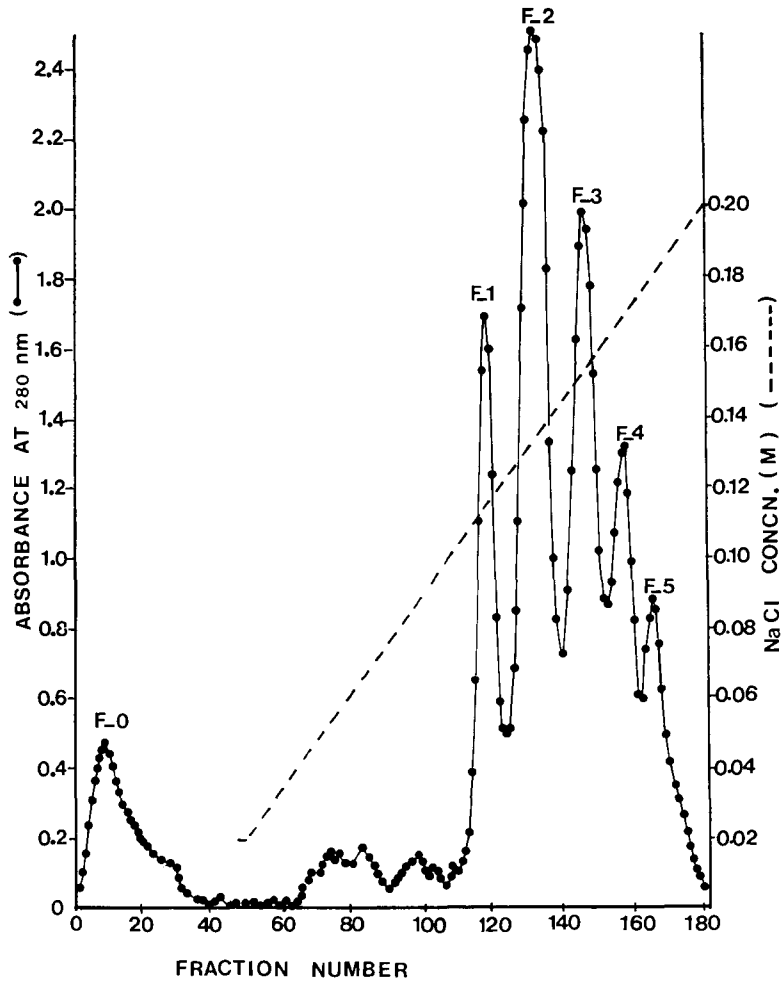


Fig. 1. DEAE-cellulose chromatographic pattern of κ -casein prepared by the modified urea-sulfuric acid method. 900 mg of κ -casein was dissolved in 45 ml of the starting buffer and reduced with 2-mercaptoethanol under nitrogen gas at 5°C for 16 hr. Reduced κ -casein was applied to a DEAE-cellulose column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

TABLE 1. Recovery of SH-modified κ -casein fractions

Peak No.	0	minor	1	2	3	4	5
Recovery %	7.86	5.68	15.58	35.58	25.05	13.47	10.32

* Peaks 1 to 5 expressed as % of the total adsorbed area.

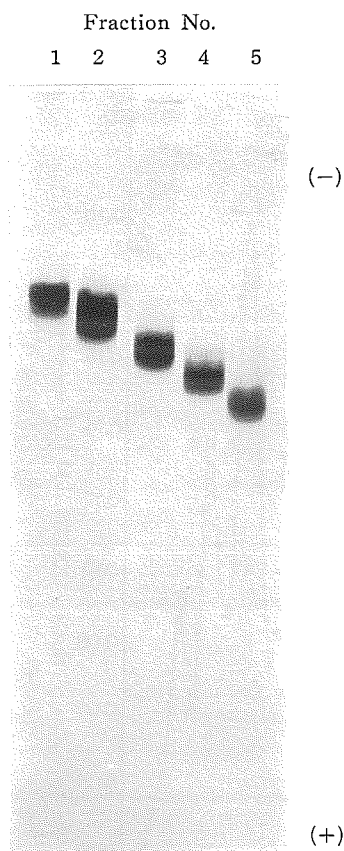


Fig. 2. Disc polyacrylamide gel electrophoretic patterns of κ -casein fractions from fraction 1 to fraction 5. 200 micrograms of protein were applied to each column. Electrophoresis was carried out in Tris-glycine buffer (pH 8.6) at 3 mA per gel for 1.5 hr. Migration was from up (cathode) to down (anode).

freeze-dried material from the fractions and the area of each peak indicated that the majority of κ -casein was adsorbed and accounted for about 90% (Table 1) of the preparation. The relative amounts of κ -casein fractions were quite reproducible on different chromatographic patterns from the same κ -casein. They were eluted from the column in the order in which they run in the polyacrylamide disc gel electrophoresis, thus the main difference between them is one of charge rather than size. The discovery of the fractions as shown in Table 1, indicates recoveries of 15.6; 35.6; 25.1; 13.5 and 10.3%, of the total adsorbed area, for the fractions 1 to 5, respectively.

The chromatogram peaks were examined by disc polyacrylamide gel electrophoresis. The electrophoretic patterns of κ -casein fractions are illustrated in Fig. 2. κ -Casein bands showed different electrophoretic mobilities. The mobilities of the protein bands were proportionally increased with the increase of elution salt concentration. The higher the salt concentration the

fraction was eluted, the faster were mobility of the bands. Fraction 1 showed the slowest mobility whereas fraction 5 was the fastest. The freeze-dried five fractions were subjected to several chemical analyses.

Phosphorus content :

Total phosphorus content of κ -casein and its fractions was determined and the results are presented in Table 2. It shows that whole κ -casein contained 1.824 μg P per mg protein while the adsorbed fractions contained 1.174 ; 1.213 ; 1.386 ; 1.038 and 1.303 μg P/mg protein for fractions 1 ; 2 ; 3 ; 4 and 5, respectively. From these results it is possible to conclude that κ -casein and all the five adsorbed fractions contain about the same, one phosphate group per molecule of protein. Moreover, the total phosphorus is distributed more or less evenly among the fractions.

From the above results it is clear that the phosphorus does not contribute to the heterogeneity of bovine κ -casein. These results are similar to that of MACKINLAY and WAKE²⁵⁾, IBUKI *et al*¹⁸⁾ and DOI *et al*⁸⁾ that κ -casein components had almost the same phosphorus content equal to one mol P per mol protein.

TABLE 2. Phosphorus content of κ -casein and its fractions

κ -Casein fractions	Whole	F-1	F-2	F-3	F-4	F-5
μg P/ mg protein	1.824	1.174	1.213	1.386	1.038	1.303
mol P/ mol "	1.18	0.76	0.78	0.89	0.67	0.84
Integer	1	1	1	1	1	1

Total nitrogen :

Total nitrogen (TN) of κ -casein and its five fractions was estimated by the micro Kjeldahl method and the results are shown in Table 3. It shows that the total nitrogen decreases with the increase of salt concentration in the gradient elution buffer. The TN percentages for κ -casein, F-1, F-2, F-3, F-4 and F-5 are 15.16, 15.12, 15.06, 14.53, 14.50 and 13.88%, respectively. The decrease in TN in the latter eluted fractions may be due to the higher carbohydrate content of these.

TABLE 3. Nitrogen content of κ -casein and its fractions

	κ -Casein fractions					
	Whole	F-1	F-2	F-3	F-4	F-5
N %	15.164	15.124	15.064	14.525	14.495	13.884

It should be stressed that nitrogen concentrations of κ -casein preparation have a wide variation from 13.6 to 15.4 gm/100 gm. The results obtained in this work showed that nitrogen content is in that range.

These results are in good agreement with the results of BEEBY⁹. During purification of the sialic acid-rich component on a DEAE-cellulose column, he obtained two fractions; one (designated κ 2-casein) contained about 15% N, 0.11% P and 0.6% sialic acid; and the other (designated κ 1-casein) contained 13.0-13.2% N, 0.96-1.04 % P and 5.9-6.3% sialic acid. Similar results also had been found by YAROSHKOVICH and YUSEF⁶² and VREEMAN *et al.*⁴⁹.

Sialic acid content:

κ -Casein carbohydrate consists of sialic acid, hexose and hexosamine. In this study, only sialic acid content of κ -casein and its fractions was estimated and the results are shown in Table 4. Whole κ -casein contains 24.57 μ g sialic acid per mg of protein, which equals to about two groups per monomeric molecule of κ -casein with MW of 20,000.

TABLE 4. Sialic acid content of κ -casein and its fractions

κ -Casein fractions	Whole	F-1	F-2	F-3	F-4	F-5
μ g/mg protein	24.57	1.97	5.32	23.42	40.95	47.29
mol/mol "	1.59	0.13	0.34	1.52	2.65	3.06
Integer	2	0	0	2	3	3

* Assuming each AcNeu group (M: 309) adds 726 (a trisaccharide unit) to the peptide molecular weight of 20,000.

The five fractions eluted by DEAE-cellulose column show a wide variation in their sialic acid content. Their sialic acid content ranged from 1.97 to 47.29 μ g per mg protein. Fraction 1 has the minimum value while fraction 5 has the maximum value. Moreover, fraction 2, 3, and 4 have 5.32, 23.45 and 40.95 μ g per mg protein, respectively.

From the above results it is possible to conclude that sialic acid in whole κ -casein is unevenly distributed among the fractions. Furthermore, the sialic acid content appeared to proportionally increase in molar increments with the increase of both sodium chloride concentration, in the second elution, and electrophoretic mobilities of the fractions. Now, it is possible to indicate that the heterogeneity of κ -casein is mainly due to the variation in sialic acid content. Also, the variations in sialic acid content are at least partly responsible for the electrophoretic differences of the five κ -casein fractions.

Stabilizing ability:

It is well known that κ -casein possesses the ability to protect other caseins against precipitation by calcium ions. Because of this unique Ca ion-stabilizing ability, κ -casein has been assigned a prominent role in determining casein micelle stability. Therefore, the ability of κ -casein fractions to

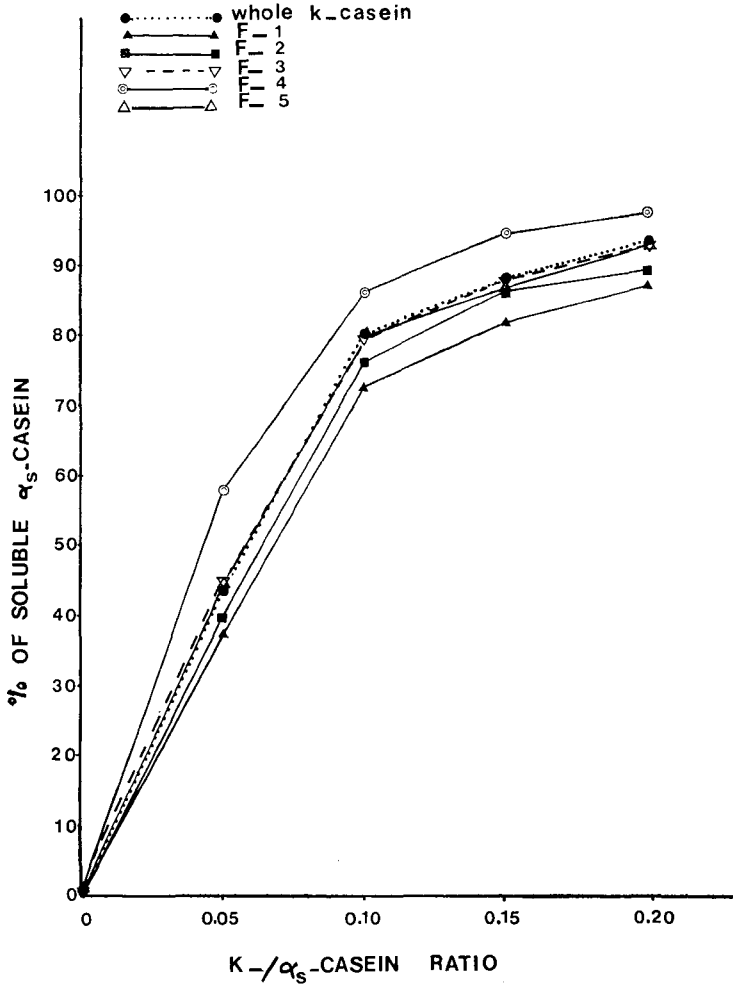


Fig. 3. Stabilization against 0.02 M Ca^{2+} of α_s -casein by reduced unfractionated κ -casein and its adsorbed five fractions. Varying volumes of κ -casein (0.5%) were added to 1 ml α_s -casein (1%) followed by adding 1 ml 0.1 M CaCl_2 . Total volume of the test mixture was adjusted to 5 ml by distilled water.

stabilize the calcium-sensitive casein (α_s) has been measured by determining the amount of soluble α_s -casein in the presence of 0.02 M CaCl_2 with various ratios of κ - to α_s -casein ranging from 0 to 0.2.

The results of the stabilizing ability are shown in Fig. 3. The data presented in this figure show that the stabilizing ability of κ -casein fractions for α_s -casein in the presence of CaCl_2 increased with the increase of κ -/ α_s -casein ratio. The higher the concentration of κ -casein in the mixture the higher is the stabilizing ability of the mixture. That phenomenon is in full agreement with the results of WAHBA and EL-HAGARAWY⁶⁰ and YAROSHKEVICH and YUSEF⁶².

In view of the differences in carbohydrate content, all κ -casein fractions had the ability to stabilize α_s -casein in the presence of CaCl_2 and quantitatively retained α_s -casein stabilizing ability with the ratio increasing. These results are similar to those of WOYCHIK⁵⁸ and MACKINLAY and WAKE²⁰. On the other hand, the stabilizing effect partially increased with the carbohydrate content increasing. Fraction 4 exhibited the greatest stabilizing ability while fraction 1 showed the least stabilizing power. It may be assumed that sialic acid has an important role in κ -casein stabilizing ability.

These results are in good agreement with the conclusion of THOMPSON and PEPPER⁴⁶, MARIER *et al.*²⁶, ROSE and MARIER³⁷ and WINTERBURN and PHELPS⁵⁷. They reported that the stabilizing ability of κ -casein seemed to be related to their sialic acid content. After removal of sialic acid, the stabilizing ability was reduced and the micellar stabilizing properties have been destroyed.

Gel Filtration of κ -casein fractions:

κ -Casein and its fractions were subjected to molecular sieving on Toyoparl HW-75 column using 0.01 M imidazole-HCl buffer at room temperature. The elution profiles of whole κ -casein and its fractions are shown in Figure 4. The gel filtration patterns indicate that whole κ -casein eluted a little faster than all the fractions. The elution peak of whole κ -casein was eluted at a volume of 48 ml while all fractions had an elution peak at elution volume of 50 ml. From these results it is possible to assume that κ -casein fractions had about the same molecular weights but those were less than that of unfractionated κ -casein complex. All fractions except fraction 4 exhibited an additional peak at elution volume of 42 ml, the additional peak of fraction 1 was the greatest one while that of fraction 3 was the lowest one.

By the column calibration technique (Fig. 5), it seems that the majority portion of all κ -casein fractions had the same molecular weight of about

545,000, while whole κ -casein had a MW of about 835,000. Moreover, the other minor portion showed a molecular weight more than 1000,000. The elution pattern of fraction 1 seemed to be more broad than others. On the other hand, the gel filtration profile of fraction 4 had a single peak with a skewed tailing edge. The pronounced tailing of the chromatographic pattern may be due to the presence of various complexes. The elution volume of fraction 3 is smaller than those of F-1 and F-5 and that of fraction 2 is the smallest. It may be assumed that the faster eluted portion consists of κ -casein fraction that polymerized through disulfide bonds^{25,44} and hydrophobic bonds⁶.

These results are in good agreement with those obtained by SEDMEROVA

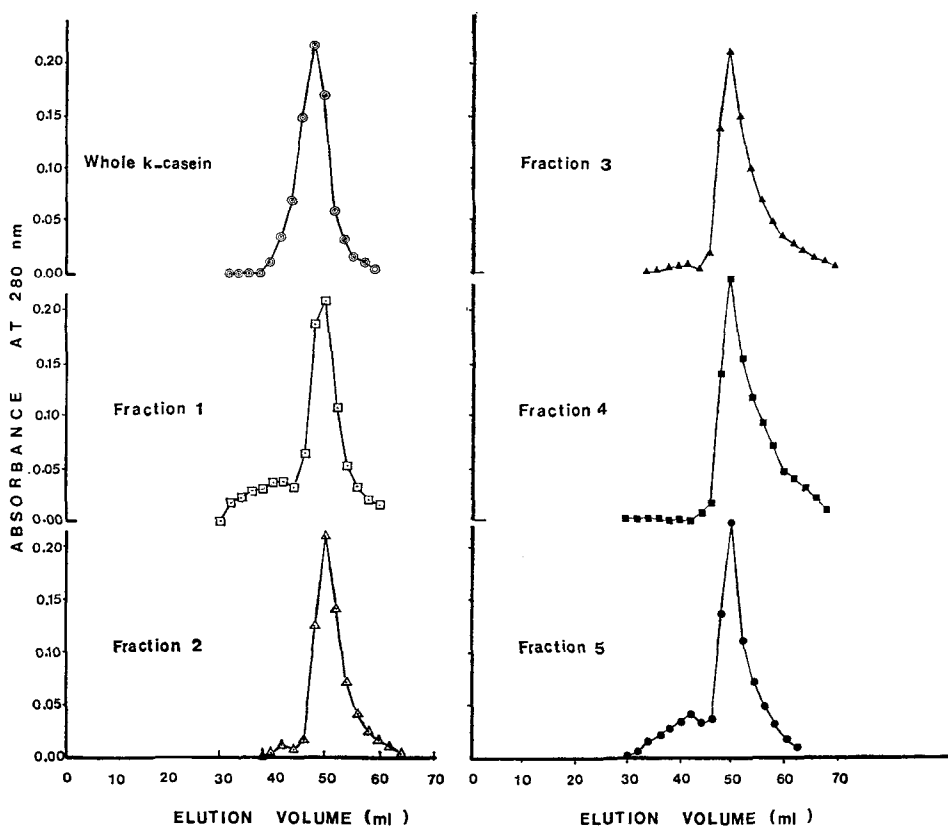


Fig. 4. Gel filtration profiles of 1 ml 0.5% κ -casein fraction on a Toyopearl HW-75 column (16 \times 435 mm) at room temperature using 10 mM imidazole-HCl buffer (pH 7.1), containing 70 mM KCl and 0.01% NaN₃. A flow rate was maintained at 25 ml per hour. One tube containing 2 ml.

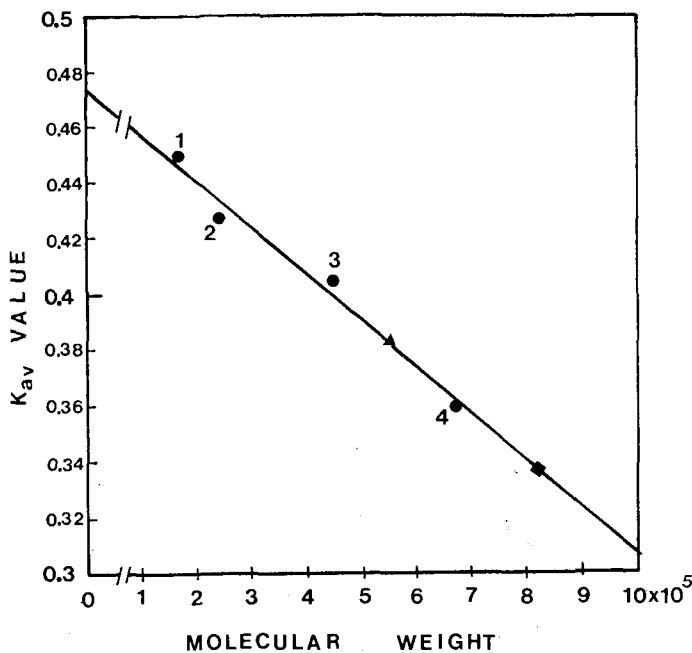


Fig. 5. Calibration curve of Toyopearl HW-75 column with proteins of known molecular weight.

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.

▲ κ -Casein fractions. ◆ Whole κ -casein.

*et al*⁴¹⁾, DOSAKO *et al*¹¹⁾, DOI *et al*^{8,9)}, WAUGH⁵³⁾, SWAISGOOD⁴³⁾ and MACKINLAY and WAKE²⁴⁾.

Discussion

Diethylaminoethyl (DEAE) cellulose has been used for the fractionation and purification of casein by several researchers^{8,9,18,26,36,54)}. The results in Fig. 1 showed that the anion exchange cellulose, with the gradient NaCl elution was very effective in separating five adsorbed distinct κ -casein fractions. These fractions could be identified approximately from the order of elution and the area under the peak.

Electrophoresis has been extensively used for the identification and characterization of the various milk proteins. The ability to isolate κ -casein components from the isoelectric purified κ -casein complex can be attributed

to the reduction of the aggregates formed by intermolecular disulfide bonds. When κ -casein has been reduced to monomeric units, normal chromatographic procedures can be applied to affect their separation and isolation. The use of gradient sodium chloride imidazole-urea system with the reduction of κ -casein has permitted the isolation of the individual κ -casein fractions. On the other hand, the reduced κ -casein fractions again undergo aggregation due to intermolecular disulfide bonding after dialysing against distilled water and freeze-drying.

Three factors are considered to affect the chromatographic pattern of κ -casein: 1- the amino acid sequence; 2- the structural feature of the carbohydrate moiety; and 3- the linkage between the peptide and sugar moieties. In case of reduced bovine κ -casein, the DEAE-cellulose chromatogram may be affected by the carbohydrate moiety.

It was pointed out that the smeared pattern, of κ -casein in gel electrophoresis even in the presence of 7 M urea, has been a matter of some concern to those investigating the heterogeneity of the caseins. It was pointed out that there were some evidence that the κ -casein fraction, alone among the casein fractions, appeared to contain cystine and possible cysteine. It has been confirmed that polydispersity due to intermolecular S-S-linkages was mainly responsible for the smeared pattern of the gel electrophoresis.

Many workers had studied κ -casein heterogeneity using either DEAE-cellulose column chromatography or several techniques of gel electrophoresis. Most of them could obtain several peaks by using a gradient elution on DEAE-cellulose or on the smeared pattern of the gel electrophoresis.

Results of this work are in good agreement with those of MACKINLAY and WAKE²⁵⁾, SCHMIDT *et al.*⁴⁰⁾, WOYCHIK *et al.*⁶⁰⁾, IBUKI *et al.*¹⁸⁾, KANAMORI *et al.*²¹⁾ and DOI *et al.*⁸⁾. They fractionated reduced κ -casein on DEAE-cellulose column into one non-adsorbed and five adsorbed fractions using NaCl gradient elution. Their gel electrophoresis patterns showed different bands and the fraction that was eluted with the higher salt concentration exhibited greater mobility.

The sialic acid content of κ -casein has been investigated by numerous researchers and appears to be close to 2.3% which is in full agreement with the results obtained in this study (Table 4). During the purification of the sialic acid-rich component on DEAE-cellulose column, BEEBY⁴⁾ detected a fraction (designated as κ_2 -casein) which contained 0.6% sialic acid; and another fraction (designated as κ_1 -casein) contained 5.9-6.3% sialic acid.

It is well known that α_s - or β -casein have 8 or 5 moles of phosphate per mole of monomer, respectively⁵⁰⁾. On the other hand, κ -casein contains

one phosphate group per mole attached with the amino acid Ser 149. By fractionation of reduced κ -casein on DEAE-cellulose, the phosphorus content was approximately evenly distributed among the fractions. The results showed that the P is dispensable as far as its ability to stabilize α_s -casein and also did not contribute to the heterogeneity. Nitrogen content of the fractions did not differ much. It decreased somewhat with the increase of sialic acid content.

The five fractions showed wide variations in both sialic acid content and the electrophoretic mobility. Although each fraction had a different electric charge, as shown from the electric mobilities, their molecular weight were similar. Therefore, it may be concluded that the microheterogeneity of κ -casein is essentially attributed to the differences of the anionically charged sialic acid contents of κ -casein fractions and can not be attributed to the molecular size.

Three prominent bands had developed from κ -casein, but only the leading one contained glycoprotein when PURKAYASTHA and ROSE³⁴ subjected κ -casein to electrophoresis on polyacrylamide gels, in the presence of urea and mercaptoethanol. They observed that the enzymic removal of sialic acid from κ -casein progressively decreased the mobility of the glycoprotein.

It is established by HUANG *et al.*¹⁷, JOLLES *et al.*¹⁹, TRAN and BAKER⁴⁸, FIAT *et al.*¹², FOURNET *et al.*^{13,14}, JOLLES and FIAT²⁰, HALBEEK *et al.*¹⁶, and SAITO *et al.*³⁹ that the carbohydrate part of κ -casein is a prosthetic group consisting of hexose, hexosamine and sialic acid in the ratio of 1 : 1 : 1. They reported that the sequence of the carbohydrate moiety of κ -casein consists of trisaccharides units which may be represented as: α -N-acetylneuraminic acid (2 \rightarrow 3 or 6)- β -galactosyl (1 \rightarrow 3) β -N-acetyl galactosaminyl. These units are attached to the peptide chain through the OH group of the threonine residue 133 by O-glycosidic linkage. A microheterogeneity has been detected at the sugar content of κ -casein²⁰.

The ability of κ -casein fraction 1, containing no sialic acid, to stabilize α_s -casein in the presence of CaCl_2 is significantly less than the other fractions that contain sialic acid. Moreover, the stabilizing effect increases with the increasing content of sialic acid (Fig. 3). These collectively show quite conclusively that sialic acid plays an important role in this function. Although the stabilizing abilities of the fractions containing sialic acid did not differ so much but the pronounced differences between fraction 1 and fraction 4 is significantly high. It may be possible to assume that, at least, sialic acid partially contributes to this function with the sequence of amino acids and the folding of the peptide chain in κ -casein molecules. This conclusion is in

accordance with the suggestion of DOI *et al*¹⁰ that the heterogeneity of κ -casein is effective on the stability of casein complex and the sugar part of κ -casein takes part in the stability. Moreover, covalently-bound carbohydrates, with their high concentration of hydroxyl groups on the peptide would increase the hydrophilic properties, and therefore the stability of the casein micelle⁵⁵.

Gel filtration, which has also been called molecular sieve chromatography, is a technique in which separation depends primarily on molecular size. Large molecules are completely excluded from the porous gel grains and emerge from the chromatographic bed at void volume. Molecules within a specific size range penetrate some gel pores and emerge later, followed by small molecules and ions that freely penetrate the gel pores. Suitable cross-linked gels were developed and it soon became apparent that gel filtration would be an efficient method for the separation and isolation of proteins and for testing homogeneity and estimating molecular size. From the results of this study it may be considered that the carbohydrate moiety of κ -casein fractions did not take part in the formation of polymers, and the difference of carbohydrate content did not lead to the difference of molecular weight of the fractions. On the other hand, whole κ -casein had the largest molecular weight in comparison to all the fractions.

Summary

Bovine isoelectric SH-reduced κ -casein was fractionated by DEAE-cellulose column chromatography, using a linear NaCl gradient, to one non-adsorbed fraction and five discrete adsorbed fractions. The adsorbed fractions showed different mobilities in disc polyacrylamide gel electrophoresis and the electrophoretic mobilities increased gradually with the increase of salt concentration. The adsorbed fractions were subjected to several analyses such as N, P, and sialic acid content. The fractions slightly varied in their N content but P content was evenly distributed among the fractions. On the other hand, the fractions had wide variations in their sialic acid content. Sialic acid content proportionally increased with the increasing of salt concentration, fraction 5 had the highest sialic acid content. All the fractions had the same molecular weight, using gel filtration technique, and the MW of the fractions was lower than that of unfractionated κ -casein. The five fractions were able to stabilize α_s -casein in the presence of CaCl_2 . The stabilizing ability partially increased with the increasing of sialic acid content. The higher sialic acid content the fractions had, the higher stabilizing abilities the fractions showed. Fraction 4, sialic acid-rich fraction, exhibited the highest stabilizing ability. The

heterogeneity of κ -casein is due to the differences in anionic sialic acid content which plays an important role in the stabilizing ability.

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