Studies on κ -Casein of Bovine Milk

Part III. Some Properties of κ -Casein and its Complex

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κ -Caseins were prepared by the calcium-ethanol method, the Sephadex method and the urea-sulfuric acid method. Some important properties of κ -caseins were investigated using isoelectric focusing, starch gel electrophoresis, ultracentrifugation, chemical analysis, stabilization test of α_s -casein, and rennin treatment. Isoelectric focusing established that κ -casein had its isoelectric point near pH 6.0 in 6 M urea, usually accompanied by a second peak around pH 5.6. Ultracentrifugation, however, showed a single peak having a $s_{20,w}$ value of 2.6~3.8 in the presence of 6 M urea and of 14.4 in the absence of such dispersing reagents. Normal contents of hexose, sialic acid, phosphorus, and nitrogen were about 1.5, 0.8, 0.2, and 14%, respectively. Relative patterns of amino acid composition were similar in all of the κ -caseins. In addition, amino acid composition in intact κ -casein and in the further purified κ -casein which formed the second peak in DEAE cellulose chromatography were almost identical, indicating that the κ -casein of the first peak is not an impurity but is one of the components which formed the original κ -casein complexes. The ability of κ -caseins to stabilize α_s -casein in the presence of calcium increased when purified by DEAE cellulose chromatography.

Methods for the preparation of κ -casein were previously examined using the criteria of yield and purity of the κ -casein obtained.¹⁾ An important property of κ -casein, heterogeneity, has been discussed in a previous paper²⁾ from the viewpoint of molecular size and charge. In the present experiment, the other interesting properties of κ -casein were examined and summarized using isoelectric focusing, starch gel electrophoresis, ultracentrifugation, chemical and amino acid analyses, and rennin treatment *etc.* κ -Caseins were compared, for

the above properties, in relation to their preparative methods. Attention is focused on the role of the weakly adsorbed component in DEAE cellulose chromatography in the construction of the κ -casein complex.

EXPERIMENTAL

Preparation of κ -casein. The calcium ethanol, urea sulfuric acid, and Sephadex methods were used to prepare κ -casein. Detailed procedures have been reported in a previous paper.¹⁾ In the present experiment, some κ -caseins were further purified by DEAE cellulose chromatography.²⁾

1) M. Kanamori, M. Miyoshi, and F. Ibuki, *J. Japan Soc. Food and Nutr.*, **22**, 639 (1969).

2) M. Kanamori, M. Miyoshi, F. Ibuki, and Z. Maki, *ibid.*, **24**, 96 (1971).

Isoelectric focusing in 6 M urea. Isoelectric focusing was carried out in a wide range ampholite (pH 3~10) according to the method described in a previous

paper.²⁾

Starch gel electrophoresis in 6 M urea. General procedures were similar to those described by R. G. Wake and R. L. Baldwin.³⁾ One exception is that a different starch concentration was used; 17 percent instead of 13 percent.

Ultracentrifugation. Five mg of κ -casein was dissolved in 1 ml of TCU buffer (tris-citrate buffer containing 6 M urea), pH 8. It was centrifuged at 52,000 rpm in a Beckman Spinco Analytical Ultracentrifuge, Model E. Temperature was kept at $25 \pm 1^\circ\text{C}$. Fifty or fifty-five minutes after the maximum speed was reached, 5 photographs were taken every 15 min for each sample. $s_{20,w}$ values were calculated based on these photographs. In one case, κ -casein prepared by the calcium-ethanol method was centrifuged at 48,000 rpm for 25 min in 0.01 M tris-citrate buffer, pH 8. The $s_{20,w}$ value of the aggregated κ -casein not unfolded by urea was also calculated.

Chemical analysis. Hexose was determined by the phenol-sulfuric acid method⁴⁾ using glucose as a standard hexose. Aminoff's thiobarbituric acid method⁵⁾ was used to determine sialic acid after the bond between sialic acid and some unknown hexose of κ -casein had been hydrolyzed at 80°C for 60 min in 0.01 N sulfuric acid. Phosphorus contents were determined by the Deniges method⁶⁾ using tin chloride as a reducing reagent following complete digestion of κ -casein in trichloroacetic acid. Nitrogen contents were measured by the micro Kjeldahl method. Tyrosine and tryptophan were measured by the ultraviolet absorption of the κ -casein solution of a known concentration in 0.1 N NaOH. This procedure is based on that described by T. W. Goodwin and R. A. Movton.⁷⁾

Amino acid analysis. Five mg of κ -casein were dissolved in 2 ml of distilled HCl and then hydrolyzed at 100°C for 22 hr and 71 hr. Air in the ampule tube containing the κ -casein solution was replaced by Argon gas

to avoid a minimum oxidation reaction. An amino acid analyzer (Hitachi KLA-3B type) was used to analyze the amino acid composition automatically. Corrections were made for variations in amino acid composition during the hydrolyzing time.

Stabilization test of α_s -casein by κ -casein. The stabilization test was performed with calcium sensitive α_s -casein (0.3%) using varying amounts of κ -casein in the presence of 0.02 M calcium chloride according to the technique of C. A. Zittle.⁸⁾

Rennin reaction. Five tenths mg of rennin powder was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.5, and 20 mg of κ -casein was dissolved in 0.5 ml of the same buffer. Twenty five hundredths ml of the κ -casein solution was mixed with 0.1 ml of the enzyme solution. The reaction was carried out at 37°C for 45 min and stopped by the addition of 0.21 g of urea. The reaction product was compared with unreacted κ -casein in the starch gel electrophoresis.

RESULTS AND DISCUSSION

Isoelectric focusing of acid casein

Four peaks were found in the isoelectric focusing of acid casein in 6 M urea. Their isoelectric points were at pH 5.0, 5.4, 6.0, and 8.2, respectively, as seen in Fig. 1. Fractions of these peaks, when analyzed by starch gel electrophoresis, were found to consist of nearly pure components as shown in Fig. 2. The

FIG. 1. Isoelectric Focusing of Acid Casein in 6 M Urea.

Column: 3×25 cm. Electrophoresis was performed at 500V for 44 hr.

3) R. G. Wake and R. L. Baldwin, *Biochim. Biophys. Acta*, **47**, 22 (1961).

4) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

5) D. Aminoff, *Biochem. J.*, **81**, 384 (1961).

6) T. Mitsui, H. Mitsuda and T. Hata, "Nogei-kagaku Jikkensho," Sangyo Tosho, 1966, p. 103.

7) T. W. Goodwin and R. A. Movton, *Biochem. J.*, **40**, 628 (1946).

8) C. A. Zittle, *J. Dairy Sci.*, **44**, 2101 (1961).

FIG. 2. Starch Gel Electrophoresis of Acid Casein and its Components Obtained by Isoelectric Focusing.

180V, 10 hr, pH 8.6, in 6 M urea.

α_s -, β -, κ -, and probably γ -caseins were well separated by this method, although the γ -casein, with little information on it, could not be identified as appearing like para κ -casein. The isoelectric points obtained in this experiment, however, deviated sharply toward the neutral or alkaline sides as compared with those obtained by Tiselius electrophoresis.⁹⁾ Although this type of deviation is naturally expected in the presence of agents such as urea, it newly proved that α_s -, and κ -caseins had clearly different isoelectric points. On the other hand, Fig. 1 shows that it is possible to estimate the relative ratio of casein components at a glance in the electrophoretic pattern. The acid casein used in this experiment is considered to be composed of 45 percent of α_s -casein, 30 percent of β -casein, 20 percent of κ -casein and 5 percent of γ -casein.

Isoelectric focusing of κ -caseins

κ -Caseins exposed to various treatments during their preparation showed that the isoelectric point of their main peaks was around pH 6.0

FIG. 3. Isoelectric Focusing of κ -Caseins in 6 M Urea.

Experimental conditions are shown in Fig. 1.

as expected from the result of isoelectric focusing of acid casein. But, Fig. 3 shows that peaks were much more asymmetrical and broader with a second peak; at pH 5.6 for κ -casein prepared by the urea-sulfuric acid method, at pH 4.9 for κ -casein isolated by the Sephadex method and at pH 5.2 for κ -casein obtained by the calcium-ethanol method.

Ultracentrifugation

κ -Casein fractionated by DEAE cellulose chromatography into two components with distinctly different molecular sizes has been reported. Results of ultracentrifugation with and without urea showed only a single peak for each κ -casein as seen in Fig. 4. These somewhat broad peaks indicate that there is some dispersion in molecular size. Therefore, no proof was obtained to show that the two components exist independently in the κ -casein complex. Effects of differences in methods for the preparation of κ -casein on its ultracentrifugal properties were found in the degree of molecular size dispersion and in the $S_{20,w}$ values. κ -Casein prepared by the urea-sulfuric acid method was interpreted to be composed of the largest and the most homogeneous

9) H. E. Swaisgood and J. R. Brunner, *J. Dairy Sci.*, **45**, 1 (1962).

FIG. 4. Ultracentrifugal Analysis of κ -Caseins.

* see Fig. 3.

molecules with $s_{20,w}$ value of 3.8. κ -Casein prepared by the calcium-ethanol method, however, seemed to have the smallest molecules with $s_{20,w}$ value of 2.6. These results are consistent with previous results of Sepharose gel filtration.²¹ As far as ultracentrifugal properties are concerned, κ -casein prepared by the Sephadex method was most dispersed or the most diffusible. κ -Casein showed an $s_{20,w}$ value of 14.4 in the absence of urea suggesting that aggregation occurred with a high degree of dispersion in molecular size.

Chemical analysis

Results of chemical analysis of κ -casein are summarized in Table I. It is noteworthy that the hexose contents of κ -casein prepared by the Sephadex method were abnormally high. The hexose contents of κ -casein, as reported by other researchers, usually range between 0.7 and 1.5%. Although no further study was made concerning the state of the carbohydrate in the κ -casein molecule, we believe that the carbohydrate was not free and was bound to the protein moiety. The reason

TABLE I. CHEMICAL ANALYSIS OF κ -CASEINS

	κ -Casein*					Method
	1	2	3	1 DEAE chromat. peak 1	1 DEAE chromat. peak 2	
Hexose (%)	1.5	1.5	15.4	1.4	1.7	Phenol-sulfuric acid
Sialic acid (%)	0.8	0.7	0.7	0.3	1.0	Thiobarbituric acid (Aminoff)
Phosphorus (%)	0.21	0.24	0.18	0.27	0.18	Deniges
Nitrogen (%)	14.3	14.3	13.5	14.6	14.1	Kjeldahl
Tyrosine (moles/20,000g)	9.5	9.8	7.5	9.7	8.4	UV absorption
Tryptophan (moles/20,000g)	0.4	0.5	0.5	0.6	0.4	UV absorption

* see Fig. 3.

for this is that κ -casein was precipitated three times by acid to remove all soluble materials before being filtered on Sephadex G-150. We reported in a previous paper²¹ that the first peak in DEAE-cellulose chromatography was composed of para-like κ -casein based on results of starch gel electrophoresis. The present chemical analysis did not provide sufficient data, except for the low content of sialic acid, to determine if it was a para κ -casein. In comparison with reliable values of amino acid composition in Tables II and III, slightly higher contents of tyrosine and slightly lower contents of tryptophan were obtained by measuring UV absorption.

Amino acid composition

There was not much difference in the amino acid composition of κ -caseins prepared by the three methods as seen in Tables II and III. κ -Casein prepared by the Sephadex method showed lower values in inverse proportion to its higher content of hexose. The relative

TABLE II. AMINO ACID ANALYSIS OF κ -CASEIN
Date are expressed as moles per 20,000g.

Amino acid	κ -Casein*			Prepared by Kalan <i>et al.</i> ¹⁰⁾
	1	2	3	
Lys	8.4	9.1	6.3	9.1
His	2.9	3.1	2.2	3.0
Arg	4.7	5.1	3.6	5.0
Asp	11.2	11.3	8.8	11.0
Thr	12.1	12.7	10.2	12.1
Ser	11.0	11.6	8.7	11.0
Glu	20.0	22.1	16.0	24.9
Pro	8.6	9.4	6.8	17.5
Gly	2.6	2.6	2.1	2.6
Ala	12.3	13.2	9.8	13.4
Cys-Cys	1.5	1.0	1.0	1.0
Val	9.1	9.4	7.5	10.2
Met	1.8	1.8	1.4	1.9
Ileu	10.2	10.7	8.2	11.5
Leu	8.1	8.5	6.2	8.7
Tyr	8.1	7.8	5.9	8.8
Phe	3.9	4.2	3.2	4.2

* see Fig. 3.

TABLE III. AMINO ACID ANALYSIS OF κ -CASEIN 1
AND ITS COMPONENTS OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY

Data are expressed as moles per 20,000g.

Amino acid	κ -Casein 1	Peak in DEAE-cellulose chromatography			
		Native		Reduced	
		2 First half	2 Second half	1	2
Lys	8.1	7.6	7.6	8.7	7.8
His	2.8	2.8	2.7	2.6	3.0
Arg	4.6	4.5	4.1	4.7	4.2
Asp	11.1	10.3	10.2	10.7	10.7
Thr	12.0	11.6	12.4	6.6	12.8
Ser	10.3	9.7	9.9	8.9	10.5
Glu	19.9	19.2	19.4	17.9	20.2
Pro	8.6	8.5	8.7	7.8	9.4
Gly	2.5	2.1	2.2	2.9	2.1
Ala	12.3	12.1	12.2	20.2	12.9
Cys-Cys	1.3	1.2	1.0	tr.	tr.
Val	8.9	8.8	8.7	7.4	9.8
Met	1.7	1.6	1.7	1.4	1.7
Ileu	9.7	9.8	9.8	8.6	10.6
Leu	8.0	7.5	7.3	9.5	7.5
Tyr	7.9	8.0	7.3	10.3	7.6
Phe	3.8	3.5	3.4	4.5	3.2

ratios of each amino acid, however, are almost the same, indicating that the peptide portions of κ -caseins are similar in nature. Data from E. B. Kalan and J. H. Woychik¹⁰⁾ are also shown for comparison. Clear differences in glutamic acid and proline were found. The remarkable decreases in the two amino acids could be due to variations in individual cows. A previous paper²¹ reported that a shoulder appeared at the second peak in DEAE-cellulose chromatography of non-reduced κ -casein. But the amino acid compositions of both the first and the second halves of peak 2 were nearly consistent with those of the second peak of reduced κ -casein, indicating that the κ -casein

10) E. B. Kalan and J. H. Woychik, *J. Dairy Sci.*, **48**, 1423 (1965).

complexes had identical peptide portions. In the amino acid composition of peak 1, threonine, serine, glutamic acid, proline, valine, and isoleucine decreased in comparison with the unfractionated κ -casein, but alanine, leucine, tyrosine, and phenylalanine increased. The amino acid composition of para κ -casein, however, is quite different from that of peak 1. Considering that no differences in amino acid composition were found between original κ -casein and the fractionated components in peak 2, it seemed that the component in peak 1 probably forms complexes, similar to those of intact κ -casein, in combination with such components as that in peak 3. In another words, it is probable that intact κ -casein contains some portions susceptible to denaturation. These portions would not usually be released, but could be dissociated into smaller units, *i.e.* by DEAE-cellulose treatment in 6 M urea.

Stabilization test of α_s -casein

κ -Casein is known to form complexes with

FIG. 5. Solubilization of α_s -Casein in the Presence of 0.02 M CaCl_2 by κ -Casein.

Casein mixture was incubated at 30°C for 15 min and centrifuged at 3000 $\times g$.

* see Fig. 3. 1': peak 2 in DEAE chromato. of κ -casein 1.

α_s -casein and to solubilize the latter in the presence of calcium. This phenomenon is called "stabilization of α_s -casein by κ -casein". Figure 5 shows that all the κ -caseins were able to stabilize α_s -casein to a moderate degree. That κ -caseins prepared by the calcium-ethanol method stabilized the greatest amount of α_s -casein is understandable if it is taken into consideration that the κ -casein is dissociated in some measure into smaller complexes, as shown in a previous paper. Dissociation into smaller units, unless it means denaturation, will increase the effective sites for the reaction with α_s -casein, so that the stability of α_s -casein increased.

Rennin treatment

Fractions in peaks 1 and 2 of the DEAE-cellulose chromatography were mixed with rennin for 45 min. The reaction products were analyzed together with unreacted fractions by the starch gel electrophoresis. Result in Fig. 6 shows that the component of peak 1 did not react with rennin indicating that the para-like component, the rennin resistant part, wrapped the surface of the κ -casein complexes. That

FIG. 6. Starch Gel Electrophoresis Showing the Results of Rennin Action on Component of κ -Casein 1 Obtained by DEAE-cellulose Chromatography.

κ -Casein (10 mg) was treated with rennin (0.05 mg) at 37°C for 45 min. Electrophoresis was performed at pH 8.6 in 6 M urea (180V, 10 hr).

is why the other minor component of peak 1, completely decomposed by rennin leaving para which moved toward the anode in starch gel κ -casein. The structures of κ -casein complexes electrophoresis, was not acted on by rennin. will be discussed again in the later papers. The component of peak 2, however, was