Studies on κ-Casein of Bovine Milk. III.

Some properties of κ-casein and its complex.

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Summary 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, stabilizing test of α_s-casein, and rennin treatment. Isoelectric focusing established that κ-casein had its isoelectric point near pH 6.0 in 6M 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 6M urea and of 14.4 in the absence of such dispersing reagents. Normal contents of hexose, sialic acid, phosphorus, and nitrogen were respectively about 1.5, 0.8, 0.2, and 14 %. Relative patterns of amino acid composition were similar in all 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 paper[2] from the viewpoints of molecular size and charge. In the present experiment, 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

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

2. *Isoelectric focusing in 6M urea.* Isoelectric focusing was carried out in a wide range ampholite (pH 3-10) according to the method described in a previous paper.[2]

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3. *Starch gel electrophoresis in 6M 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.

4. *Ulracentrifugation.* Five mg of κ-casein was dissolved in 1 ml of pH 8, TCU buffer (tris-citrate buffer containing 6M urea). It was centrifuged at 52,000 r.p.m. in a Beckman Spinco Analytical Ultracentrifuge Model E. Temperature was kept at 25±1°C. Fifty or fifty-five minutes after the maximum speed was reached, 5 photographs were taken every 15 minutes 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 r.p.m for 25 minutes in 0.01M tris-citrate buffer, pH 8. The $s_{20,w}$ value of the aggregated κ-casein not unfolded by urea was also calculated.

5. *Chemical analysis.* Hexose was determined by the phenol sulfuric acid method[4] using glucose as a standard hexose. Aminoff's thiobarbituric acid method[5] 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 minutes in 0.01N sulfuric acid. Phosphorus contents were determined by the Deniges method[6] using tin chloride as a reducing reagent following complete digestion of κ-casein in trichloro-acetic acid. Nitrogen contents were measured by the micro Kjeldahl method. Tyrosine and tryptophan were measured by the ultra violet absorption of the κ-casein solution of a known concentration in 0.1N NaOH. This procedure is based on that described by T.W. Goodwin and R.A. Movton.[7]

6. *Amino acid analysis.* Five mg of κ-casein was dissolved in 2 ml of distilled HCl and then hydrolyzed at 100°C for 22 hours and 71 hours. Air in the ample tube containing the κ-casein solution was replaced by Argon gas to avoid a minimum oxidation reaction. An amino acid analyzer (Hitachi KLA −3A type) was used to automatically analyze the amino acid composition. Corrections were made for variation in amino acid composition during the hydrolyzing time.

7. *Stabilizing test of α_S-casein by κ-casein.* The stabilization test was performed with calcium sensitive α_S-casein (0.3%) using varying amount of κ-casein in the presence of 0.02M calcium chloride according to the technique of C.A. Zittle.[8]

8. *Rennin treatment.* Five tenths mg of rennin powder was dissolved in 1 ml of 0.1M 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 minutes and stopped by the addition of 0.21g of urea. The reaction product was compared with unreacted κ-casein in the starch gel electrophoresis.

Results and Discussion

1. *Isoelectric focusing of acid casein.*

Four peaks were found in the isoelectric focusing of acid casein in 6M 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

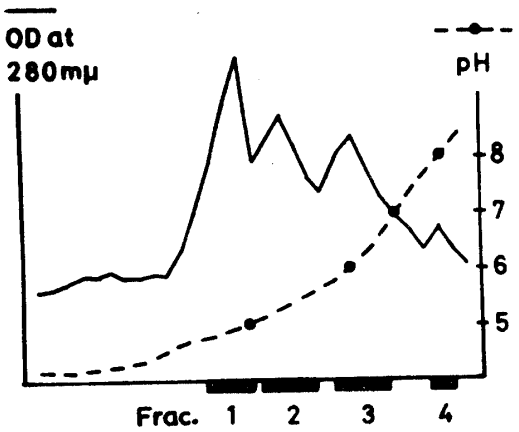

Fig. 1. Isoelectric focusing of acid casein in 6M urea.
Column: 3×25 cm. Electrophoresis was performed at 500 V for 44 hrs.

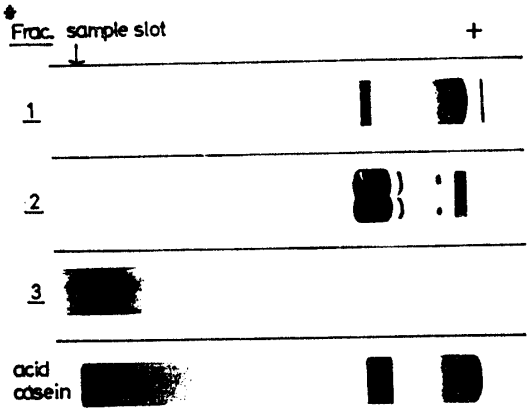

Fig. 2. Starch gel electrophoresis of acid casein and its components obtained by isoelectric focusing.
180V, 10hrs, pH 86, in 6M urea.
※See Fig. 1.

found to consist of nearly pure components as shown in Fig. 2. The 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 side 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.

2. *Isoelectric focusing of κ-caseins.*

κ-Caseins exposed to various treatments during their preparation showed that the isoelectric points of their main peaks were around pH 6.0 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-casin isolated by the Sephadex method and at pH 5.2 for κ-casein obtained by the calcium ethanol method.

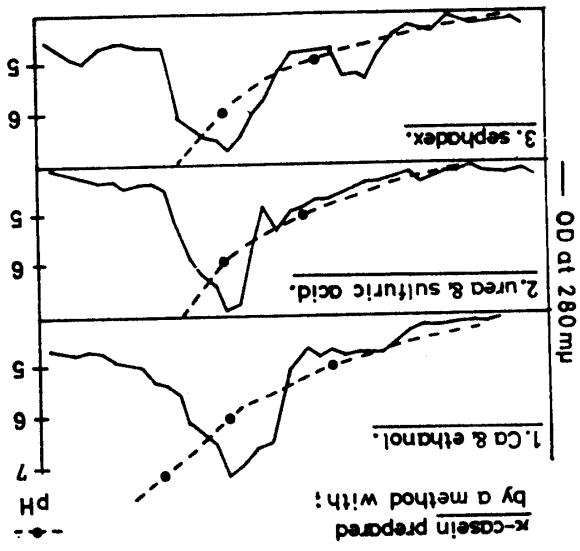

Fig. 3. Isoelectric focusing of κ-caseins in 6M urea.
Experimental conditions are shown in Fig. 1.

3. *Ultracentrifugation.*

κ-Casein fractionated by DEAE cellulose chromatography into two components with distinctly different molecular sizes has been reported.[2] Results

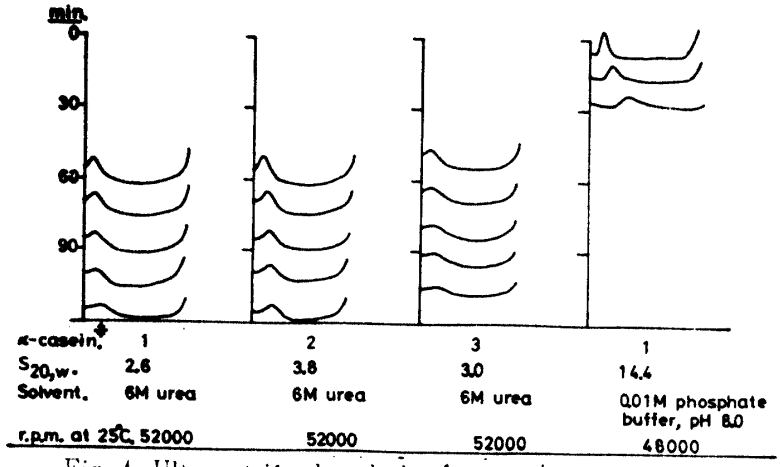

Fig. 4. Ultracentrifugal analysis of κ-caseins.
*see Fig. 3.

of ultracentrifugation with and without urea showed only a single peak for each κ-casein as seen in Fig. 4. Somewhat broad, these 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 ultracentifugal properties were found in the degree of molecular size dispersion and in $s_{20,w}$ values. κ-Casein prepared by the urea sulfuric acid method was interpreted to be composed of the largest and the most homogeneous 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 filtaration.[2] 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.

4. *Chemical analysis.*

Results of chemical analysis of κ-casein are summarized in Table 1. It is noteworthy that the hexose contents of κ-casein prepared by the Sephadex method

Table 1. Chemical analysis of κ-caseins. * see Fig. 3.

κ-casein*	1	2	3	1 DEAE chromato. peak 1	1 DEAE chromato. peak 2	Method
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/20000g)	9.5	9.8	7.5	9.7	8.4	UV absorption
Tryptophan (moles/20000g)	0.4	0.5	0.5	0.6	0.4	UV absorption

were abnormally high. The hexose contents of κ-casein, as reported by other reseachers, usually range between 0.7 and 1.5 percent. 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 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 paper[2] 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 2 and 3, slightly higher contents of tyrosine and slightly lower contents of tryptophan were obtained by measuring ultra violet absorption.

5. *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 2 and 3. κ-Casein prepared by the

Table 2. Amino acid analysis of κ-casein. Data are expressed as moles per 20000g. *see Fig. 3.

amino acid	κ-casein*			10) prepared by Kalan et al
	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

Table 3. Amino acid analysis of κ-casein 1 and its components obtained by DEAE cellulose chromatography. Data are expressed as moles per 20000g.

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

Sephadex method showed lower values in inverse proportion to its higher content of hexose. The relative 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. Woychik[10] 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 paper[2] 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 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 dfferent 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 other words, it is probable that intact κ-casein contains some portions susceptible to denaturation. These portions would not usually be released, but could be disintegrated into smaller units, i. e. by DEAE cellulose treatment in 6M urea.

6. *Stabilizing test of α_s-casein.*

κ-Casein is known to form complexes with α_s-casein and to solubilze the latter in the presence of calcium. This phenomenon is called "stabilization of α_s-casein by κ-casein". Fig. 5 shows that all the κ-caseins were able to stabilize α_s-casein to a moderate degree. That κ-casein 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.[2]. 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 increases.

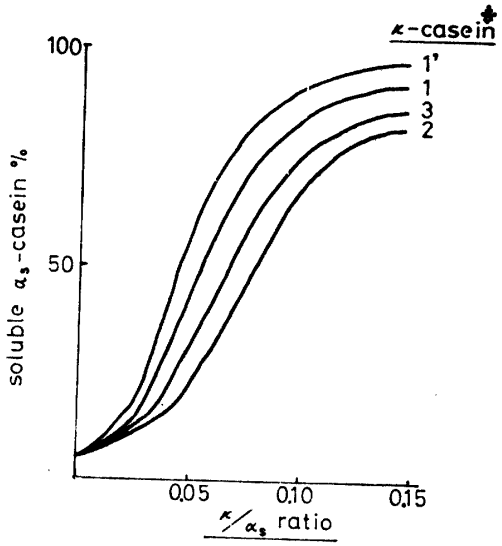

Fig. 5. Solubilization of α_s-casein in the presence of 0.02M $CaCl_2$ by κ-casein. Casein mixture was incubated at 30°C for 15 min and centrifuged at 3000×g.
※see Fig. 3. 1': peak 2 in DEAE chromato. of κ-casein 1.

7. *Rennin treatment.*

Fractions in peaks 1 and 2 of the DEAE cellulose chromatography were mixed with rennin for 45 minutes. 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 resistent part, wrapped the surface of the κ-casein complex. That is why the other minor component of peak 1, which moved toward the anode in the starch gel electrophoresis, was not acted on by rennin. The component

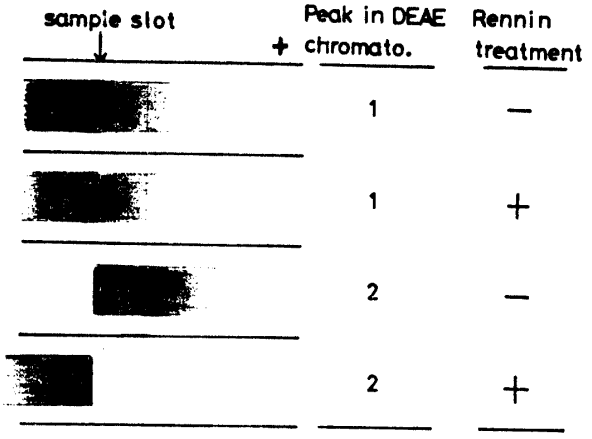

Fig. 6. Starch gel electrophoresis showing the results of rennin action on component of κ-casein 1 obtained by DEAE cellulose chromatography.
κ-Casein (10mg) was treated with rennin (0.05mg) at 37°C for 45 minutes. Electrophoresis was performed at pH 8.6 in 6M urea (180V, 10hrs).

of peak 2, however, was completely decomposed by rennin leaving para κ-casein. The structure of κ-casein complexes will be discussed again in the coming papers.

要旨：κ-カゼインを三種の方法で調製し，電気泳動，超遠心分析，α_S-カゼイン安定化力等について調べた。尿素中における等電点はpH6.0であり，α_S-カゼインとは違うことが明確になった。$s_{20,w}$は尿素中で2.6～3.8であり尿素が存在しないとpH 8.0で14.4を示した。ヘキソースは1.5%，シアル酸は0.8%，リンは0.2%，チッ素は14%程度であった。DEAEセルロースクロマトグラフィーによって分画される前後でアミノ酸組成には変化がなかったが，α_S-カゼイン安定化力は分画後増大する傾向にあった。

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