# Studies on κ-Casein of Bovine Milk. I.
# Purity of κ-casein prepared by several different methods.

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**Summary** A comparison of five procedures for preparing κ-casein from the acid casein of bovine milk was made based on the criterion of purity using Sephadex gel filtration and starch gel electrophoresis.

There were no significant differences in yields among the five procedures, by which two to three grams of κ-casein were obtained from 43 grams of acid casein. But the κ-casein prepared using the calcium ethanol method described by Mckenjie and Wake or by the Sephadex method described by Yaguchi et al. was very pure. It was also shown that it is possible to prepare reasonably pure κ-casein using the trichloroacetic acid method, described by Swaisgood and Brunner, combined with Sephadex treatment, and using the urea sulfuric acid method described by Zittle and Custer when temperature was strictly controlled in the precipitation step of κ-casein by ammonium acetate.

κ-Casein, one of the components of bovine milk casein occupying about 15 percent of whole casein, is composed of polypeptides connected by S-S bonds and has an important function in preventing $\alpha_s$-casein from precipitating in the presence of calcium.[1] When rennin acts on whole casein, κ-casein is specifically digested and loses its stabilizing ability resulting in the precipitation of casein aggregates.[2] Recent studies[3] on κ-casein have shown several components in the gel electrophresis of reduced κ-casein. Structural analyses of these components determined the C-terminal amino acid and the sequence of some amino acids succeeding it.[4] Other interesting discoveries[5] concerning κ-casein are of no direct concern of carbohydrate moiety of the κ-casein molecule with stabilizing function, of the presence of heterogeneous products of rennin digestion due to different origins,[6] and of the presence of phenotype A and B which differ in only one of the components of κ-casein.[7] Because it is necessary to obtain pure κ-casein for the fundamental studies mentioned above, the present experiment was performed to compare five representative methods for the preparation of κ-casein by main citerion of its purity.

## Experimental methods

*1. Preparation of κ-casein.* κ-Casein was prepared from acid casein using the five procedures described in Figs. 1, 2, 3, 4, and 5.[8, 9, 10, 11, 12]

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Received on July 31, 1971

1. Ca-EtOH Method (Mckenjie & Wake)

Acid casein
- dissolve in water (1.5 L, pH 7.5)
- add 4 M $CaCl_2$ (0.15 L, pH 6.5) 35°C

Sup.
- add 1.5 M K-oxalate (0.5 L) 0°C

Sup.
- add $Na_2SO_4$ 500 g

Ppt.
- dissolve in water or 6 M urea sol.
- dialyze against 0.005 M NaCl
- add equal vol. of EtOH & 2 M $AcONH_4$

Ppt.
- dissolve in 6 M urea sol.
- dialyze & freeze-dry

Fig. 1. Method for the preparation of κ-casein.

2. TCA method (Swaisgood & Brunner)

Acid casein
- dissolve in 6.6 M urea (2 L)
- dilute to 3.3 M, pH 4.8

Ppt.
- dissolve in 6.6 M urea
- dilute to 4.0 M

Ppt.
- dissolve in 6.6 M urea
- add TCA (12 % w/w), 3°C

Sup.
- pH 7.0
- dialyze
- make 0.25 M $CaCl_2$, 3°C → 30°C.

Sup.
- dialyze
- adjust pH to 11.3 & back to 4.4

Ppt.
- dissolve in water
- dialyze & freeze-dry

Fig. 2. Method for the preparation of κ-casein.

3. Urea-sulfuric acid method (Zittle & Custer)

Acid casein
- dissolve in 6.6 M urea (1 L)
- add 7N $H_2SO_4$ (200 ml) & $H_2O$ (2 L)

Sup.
- add $(NH_4)_2SO_4$ 132 g/L

Ppt.
- dissolve in water
- dialyze
- add 2 vol. of EtOH
- add 1 M $AcONH_4$ until ppt. forms

Ppt.
- dissolve in water
- dialyze & freeze-dry

Fig. 3. Method for the preparation of κ-casein.

4. Sephadex method(Yaguchi, Davies & Kim)

Acid casein
- dissolve in water
- make 0.25 M $CaCl_2$ soln.

Sup.
- dialyze & freeze-dry

Fraction S
- Sephadex G-150
  - 2.5 x 95 cm, TCU buffer pH 8.6

Pooled fractions of the first peak
- dialyze & freeze-dry

Fig. 4. Method for the preparation of κ-casein.

5. DEAE-cellulose method (Hill)

Acid casein

Fraction S

DEAE-cellulose chromatography
- 2.5 x 15 cm
- elution

| | *buffer(ml) A | B |
|---|---|---|
| stage 1 | 100 | 0 |
| stage 2 | 100 | 100 |

Pooled fractions of the second peak
- dialyze & freeze-dry

*buffer
A : 0.05 N AcOOH, pH 6.25 B : 0.1 N AcOOH containing $CaCl_2$(0.5 M), pH 4.5

Fig. 5. Method for the preparation of κ-casein.

*2. Modification of methods for the preparation of κ-casein.* Depending upon the results of a purity examination by gel filtration and by starch gel electrophoresis, some steps in the above preparative methods were modified, but not to such a degree that the fundamental principles involved in the original methods were changed. In Mckenjie and Wake's method, the ammonium acetate precipitation step was repeated three times. In Swaisgood and Brunner's method, the final preparation was treated with Sephadex G-150 gel filtration. In Zittle and Custer's method, the temperature at the point of ammonium acetate precipitation in 75% alcohol was strictly controlled at 25°C.

*3. Gel filtration on Sephadex G-150.* κ-Casein, 100 mg, was dissolved in 5 ml of TCU buffer, pH 8.6 (0.05M tris-citrate buffer containing 6M urea) and was applied to a column of Sephadex G-150 (2.5×95cm) equilibrated with the buffer described above. Flow rate was kept at 10 ml per hour and individual fraction volume was made to 7.5ml.

*4. Starch gel electrophoresis.* The general procedure and apparatus were as described by Wake and Baldwin.[13] Urea was added to the heated TCU buffer (0.076M, pH 8.6, containing 12% starch) to make the concentration 7M. Starch gel was formed after cooling. One percent κ-casein solution in TCU buffer was applied to a sample slot and electrophoresis was carried out at 180 V for about 10 hours using 0.3M borate buffer, pH 8.6, as the electrode solution. Saturated amido black solution was used for dying and 10% acetic acid in 50% alcohol was used for decoloring.

## Results and discussion

*1. Yield.*

The yields of κ-casein isolated from 43 grams of acid casein using various methods are shown in Table 1. Though there is no significant difference in yield, a great deal of κ-casein was lost during preparation. The yield of 3 grams or so is about 30 to 40 percent of the theoretical value.

Table 1. Yield of κ-casein from 43 gram acid casein

| κ-Casein prepared by | Yield (g) |
|---|---|
| 1. Mckenjie & Wake's method | 2.9 |
| 2. Swaisgood & Brunner's method | 2.9 |
| 3. Zittle & Custer's method | 3.5 |
| 4. Yaguchi, Davies & Kim's method | 2.5 |
| 5. Hill's method | 2.2 |

*2. Purity.*

Though starch gel electrophoresis has generally been used to examine the purity of κ-casein, it is inconvenient because of the slow mobility and abnormally spread band often shown by κ-casein in this electrophoresis. That is why gel filtration was used here in combination with starch gel electrophoresis. κ-Casein is a large molecule of approximately 14 S, while other caseins such as $\alpha_S$- and β-caseins are of about 2 S. In addition, these caseins are apt to form complexes with each other so that it is necessary to dissociate κ-casein from the others before its isolation. Urea is often used to disperse casein complexes. Yaguchi et al. succeeded in separating κ-casein from other caseins using a gel filtration technique in 6M urea. Results of gel filtration of the κ-caseins 1-5 are

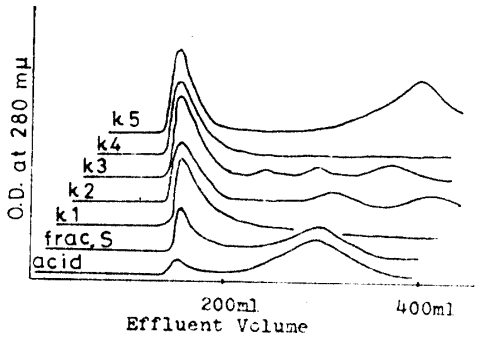


Fig. 6. Gel filtration patterns of casein on Sephadex G-150

acid: acid casein, frac. S: soluble casein in 0.25 M $CaCl_2$ solution, κ 1-5: see Table 1. 100mg casein was applied on a column (2.5×95 cm) and eluted with 0.05 M tris-citrate buffer (pH 8.6) containing 6 M urea.

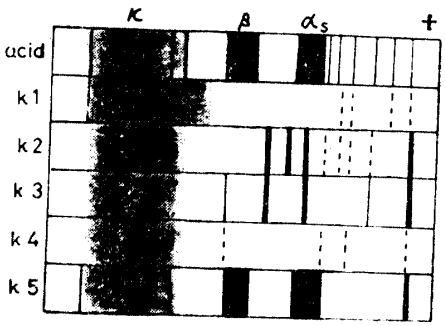


Fig. 7. Starch gel electrophoresis of casein in 7 M urea

acid, κ 1-5: see Table 1
Electrophoresis was performed in 0.076M tris-citrate buffer (pH 8.6) containing 7 M urea. 180 voltages were applied for 10 hours at 5°C.

shown in Fig. 6. The first peak around 150 ml effluent volume is that of κ-casein. When compared with patterns of acid casein and fraction S, it is clear how well these κ-caseins were isolated. Judging from the elution pattern of acid casein, the percentage of κ-casein in it coincides with the 15 percent estimated by electrophoresis. When the microheterogeneity of κ-casein is a central problem, one critical point is that the recovery of κ-casein from acid casein is at best 40 percent. Those peaks following the first in Fig. 6 are thought to be due to some other casein or impurities. κ-Casein 1 and 4 are almost pure, but may be contaminated by a negligible amount of impurities which appear as a shoulder on the first peak. On the other hand, κ-casein 2, 3, and 5 are, having the third and the fourth peaks in addition, supposed to contain 20-30 percent impurities. The pattern of κ-casein 4 shows that κ-casein obtained by Sephadex method reproduces the same filtration pattern with no degradated product when gel-filtrated again. Impurities of three kinds appeared as a tailing part of the first peak, as a sub-peak around the effluent volum 300 ml and as another sub-peak around 400 ml. The first impurity could not be completely removed from κ-casein 1 and 4. All impurities remained in κ-casein 2 and 3, and a large amount of the third impurity remained in κ-casein 5.

Results of starch gel electrophoresis are shown in Fig. 7. Considering that κ-casein is colored by amido black less intensively than it should be because of its spread band, κ-casein 2, 3, and 5 contain more than 20 percent of impurities which appear as barely visible bands. These results are similar to those obtained by gel filtration. For some unknown reason, κ-casein 1 was less apt to be colored. κ-Casein 2 and 3 contain some unknown protein that runs farthest toward the anode. This unknown protein was also found by Swaisgood et al. and is thought to be produced during acid treatment in the preparation. A very small amount of $\alpha_s$-casein was found in κ-casein 4, but this simple method turned out to be very effective in purifying κ-Casein. κ-Casein 5 contained large amounts of impurities, which means the DEAE cellulose method needs a great deal of improvement.

*3. Modification of preparative methods.*

This investigation proved that Mckenjie and Wake's method was the most suitable for preparing $\kappa$-casein. In this method, $\kappa$-casein which was once precipitated by ammonium acetate out of 50% alcohol was again dissolved in water at pH 7.5, then it was repeatedly precipitated in the same way. This step was repeated three times here until pure $\kappa$-casein was obtained. Fig. 6 shows results of starch gel electrophoresis of proteins, which were soluble at each of the precipitation steps using ammonium acetate, and results for the finally precipitated protein. The results distinctly indicate that this precipitation step plays a very important role in the purification of $\kappa$-casein. Most of the $\beta$-casein was removed at the second step, while most of the $\alpha_s$-casein was removed at the third step. Since there was an increasing loss of $\kappa$-casein at the third step, this precipitation treatment should not be repeated more than three times to avoid a large loss of $\kappa$-casein at this step. It was difficult to remove all the $\alpha_s$-casein by Swaisgood and Brunner's method. As removal of $\alpha_s$-casein by 0.25M $CaCl_2$ was incomplete due to permanent turbidity produced at this treatment, gel filtration with Sephadex G-150 was used for further purification of $\kappa$-casein 2. The result shown in Fig. 9 clearly indicates that most of the impurities were removed with only one impurity left at the anode side. The remaining impurity seems to be a very large molecule, and its origin and characters should be studied further. Care should be taken to Zittle and Custer's method, the most popular one, in order to exclude even a minute impurity. Repeating the acid treatment which potentially denaturates $\kappa$-casein exposed to low pH is undesirable. Therefore, this method was modified so that the temperature was strictly controlled at 18°C and 25°C in the ammonium acetate precipitation step in 75% alcohol. Impurity of the $\kappa$-casein obtained was checked with starch gel electrophoresis and gel filtration as shown in Fig. 10. Yields were higher, but the purity of $\kappa$-casein was quite lower at 18°C. As temperature control is an important factor in purifying $\kappa$-casein by ammonium acetate precipitation, we recommend that the temperature be kept at 25°C. In addition, the concentration of alcohol should be 50 percent rather than 75 percent in this step to increase the efficiency of this step for purification. In Yaguchi, Davies, and Kim's method, $\kappa$-casein is considered to be completely dispersed and dissociated by the presence of urea, which brings about the ease with which pure $\kappa$-casein can be obtained. The only disadvantage of this Sephadex method is that it would not be useful for mass-preparation. There was difficulty in modifying Hill's method in spite of the possibility of improving DEAE cellulose chromatography in combination with some suitable dispersing agents. In fact, Rose

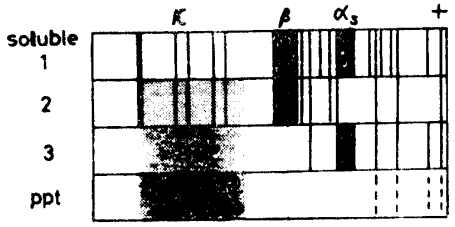


Fig. 8. Effect of ammonium acetate precipitation on the starch gel electrophoresis pattern of $\kappa$-casein

$\kappa$-Casein was prepared by Mckenjie and Wake's method. As to other experimental conditions, see Fig. 2. Soluble 1,2 and 3 are the soluble caseins at the first, the second and the third precipitation step by ammonium acetate, respectively. Ppt is the finally precipitated casein at the third step.

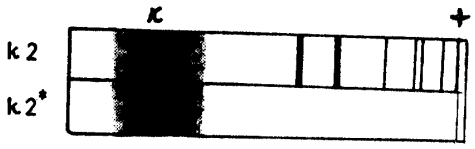


Fig. 9. Effect of Sephadex treatment on the starch gel electrophoresis pattern of κ-casein

*: further purified by Sephadex G-150 gel filtration. κ-Casein was prepared by Swaisgood and Brunner's method. As to other experimental conditions, see Fig. 1 and 2.

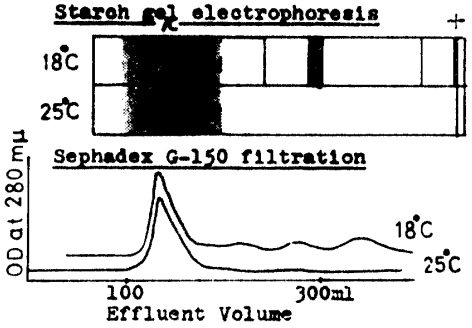


Fig. 10. Effect of temperature on the purity of κ-casein

κ-Casein was prepared by Zittle and Custer's method. 18°C and 25°C indicate κ-casein precipitated from 75% ethanol solution by ammonium acetate at each temperature.

et al.[14] succeeded in isolating casein components by alkylating them before DEAE cellulose treatment. Other interesting observations worth mentioning are the eminently yellow color of κ-casein 4, the presence of an unknown component in κ-casein 2 and 3 which runs farthest toward the anode in starch gel electrophoresis, and the slightly faster mobility of κ-casein 1 in starch gel electrophoresis. These observations point up the necessity of further investigation on the chemical composition and structural properties of κ-caseins obtained by various methods.

要旨：五種類の κ-カゼイン調製法を比較検討した結果，収量の点では各方法とも酸カゼイン 43gより 2ないし 3g で有意の差は認められなかった。一方，セファデックスゲルろ過とデンプンゲル電気泳動によって純度を比較したところ Mckejie らのカルシューム・エタノール法と Yaguchi らのセファデックス法が好結果を与えた。しかし，純度の点で問題のあった Swaisgood らの TCA 法，Zittle らの尿素硫酸法でも，各々セファデックス処理を加えること，酢酸アンモンによる沈殿形成時の温度条件を厳守することにより高純度の κ-カゼインを調製しうることを認めた。

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