Preparation of κ-Casein by Gel Filtration

MAKOTO YAGUCHI, D. T. DAVIES, and Y. K. KIM
Division of Biosciences
National Research Council, Ottawa, Canada

Abstract

A procedure for the preparation of pure κ-casein by Sephadex gel filtration is described. Unreduced casein is filtered through a 2.5- by 95-cm column of Sephadex G-150 equilibrated with 0.005 M tris-citrate buffer, pH 8.6, containing 6 M urea. κ-Casein is excluded from the gel and emerges as a sharp peak at the void volume; all other caseins are retarded by the gel. The method is particularly suitable for preparing pure κ-casein in good yield from small quantities of whole casein, but larger amounts of κ-casein are readily prepared. The method is also suitable for the purification of κ-casein-rich preparations.

In milk micelles, and in preparations such as acid-precipitated whole casein, κ-casein forms a complex with α_s- and β-caseins. Urea dissociates this complex (8), the α_s- and β-caseins being converted into monomeric forms while κ-casein remains as relatively large aggregates with intermolecular disulfide bonding (4, 6, 13). Yaguchi and Tarassuk (18) observed that when a solution of acid casein at pH 8.6 passed through a column of Sephadex G-100, which had been equilibrated with 6 M urea at pH 8.6, relatively pure κ-casein was eluted at the void volume of column. Also, Nakai et al. (5) report that κ-casein was eluted solely at the void volume when unreduced κ-casein was filtered through columns of Sephadex G-200 equilibrated with either tris-hydrochloric acid-urea (pH 7.6) or acetic acid-urea, or urea-sodium chloride, solutions. These observations suggested that gel filtration might prove useful in the preparation of κ-casein. The present paper presents a method for the routine preparation of pure κ-casein from acid casein, or from κ-casein-rich materials, which is convenient and produces an undegraded product with little or no contaminating protein.

Methods and Materials

Preparation of whole caseins. Various fresh skimmilks from individual cows and fresh herd milk were used, but all data reported in detail were obtained in tests with milk, from a single Ayrshire cow, containing α_s-casein B, β-casein A, and κ-casein A.

Acid casein was prepared at pH 4.6 and washed with water. Soluble casein was prepared according to von Hippel and Waugh (15), except that the micellar casein was brought into solution by adding a mixture of di- and tetrasodium salts of ethylenediaminetetraacetic acid (EDTA) to chelate the calcium.

Preparation of κ-casein-rich materials. Three K-casein-rich caseins (designated S, H, and Z) were made from either acid casein or first-cycle soluble casein. Casein S was Fraction S of Waugh and von Hippel (17), Casein H was prepared according to Hill (1) and Hill and Hansen (2), but without the final step involving DEAE-cellulose chromatography, and Casein Z was made according to Zittle and Custer (20) but without the final ethanol treatment.

Sephadex gel filtration. Sephadex G-150 (Pharmacia, Uppsala, Sweden), which had been freed of fine material after soaking in distilled water, was equilibrated with 0.005 M tris-citrate buffer (pH 8.6) containing 6 M urea (TCU buffer), which had been filtered through a short column of DEAE-cellulose to remove colloidal impurities. A slurry of the equilibrated Sephadex was packed, at room temperature, into a Sephadex Laboratory Column (K25/100, Pharmacia, Uppsala, Sweden) to give a gel column 2.5 by 95 ± 2 cm, a sample applicator was placed on top of the gel, and column consolidated by passing through it, under a pressure head of 30 cm of water or less, a volume of TCU buffer equivalent to at least twice the gel bed volume.

The casein sample (up to 2 g) to be fractionated was dissolved in 25 ml TCU buffer and centrifuged for 20 minutes at 2 C and 90,000 × *g* (Spinco Model L, Rotor no. 30). Centrifugation separated the contents of the tubes into an upper turbid layer, which was mainly lipid, a transparent liquid layer, and a small pellet of sedimented material. The transparent liquid was collected and dialyzed overnight at 4 C against TCU buffer. A volume of 10 ml was then applied to the Sephadex gel and eluted with TCU buffer under a pressure head of not more than 30 cm, with a resultant flow rate of 20-40 ml/hr. Five hundred milliliters of effluent were collected, in

Received for publication September 26, 1967.
N.R.C. no. 9981.

10-ml fractions, and the absorbance of the fractions was measured at 280 mμ. Pooled effluent fractions were dialyzed for at least 12 hours against 0.005 M sodium chloride and then concentrated by ultrafiltration (14) to a volume of 5-10 ml. The concentrated fractions were further dialyzed exhaustively against repeated changes of the dilute sodium chloride, and freeze dried.

Preparation of rennin-treated caseins. A 1% solution of casein in 0.1 M tris-citrate buffer (pH 6.6) was incubated with rennin (1 μg rennin/mg casein) for 15 minutes at 35 C. An equal volume of urea-containing buffer (8 M urea) was then added to inactivate the enzyme and the mixture dialyzed overnight at 4 C against 0.076 M tris-citrate buffer (pH 8.6) containing 8 M urea and 0.04 M β-mercaptoethanol.

Polyacrylamide gel electrophoresis. Polyacrylamide gels were prepared by diluting 30 ml of a 20% solution of Cyanogum 41 (Fisher Scientific Co.) to 88 ml with distilled water, deaerating the solution under suction, adding 230 μl of Cyanogum 41 catalyst (β-dimethylaminoproprionitrile) and 12 ml of 4% ammonium persulfate, and pouring the mixed solution immediately into the mold described by Purkayastha and Rose (11), then allowing about one hour for polymerization. The gel was removed from the mold, washed with several changes of distilled water, and equilibrated against 0.076 M tris-citrate buffer (pH 8.6) containing 8 M urea and 0.04 M β-mercaptoethanol for at least 48 hours. The gel was then superficially dried with absorbent paper, taped to a support (11) and trimmed so that about 5 mm were left protruding at the end nearer the sample slots. About 500 μg of protein, dissolved in the equilibrating buffer, was applied, and the surface of the gel sealed with molten petrolatum. During electrophoresis the gel was held vertically and the discontinuous buffer system of Poulik (9) was used, with bridge vessels containing 0.3 M boric acid (titrated to pH 8.6 with sodium hydroxide) and electrode vessels, 0.1 M sodium chloride. With the cathode at the top of the apparatus, a constant current of 15 ma was applied for about ten hours. After electrophoresis, the gel was stained with 1% Amido Black in methanol, water, acetic acid (5:10:1 by volume) and the excess of dye then removed by repeated washings, over several days, with the same mixed solvent (10).

Sialic acid determination. Sialic acid was determined by a modification of the Warren method (16) as used by Marier et al. (3).

Results

In preliminary tests, columns of various dimensions prepared with Sephadex G-100, G-150, and G-200 were compared. With a 2.5- by 95-cm column, Sephadex G-100 excluded κ-casein plus some other casein, while Sephadex G-200 allowed some κ-casein to penetrate the gel. Sephadex G-150, on the other hand, excluded only κ-casein, and κ-casein therefore eluted as a sharp peak at the void volume of the column.

Gel filtration of all casein samples tested yielded two distinct fractions; two representative elution curves are shown in Figure 1. In each instance, Fraction I was not retarded by the column, while Fraction II was consider-

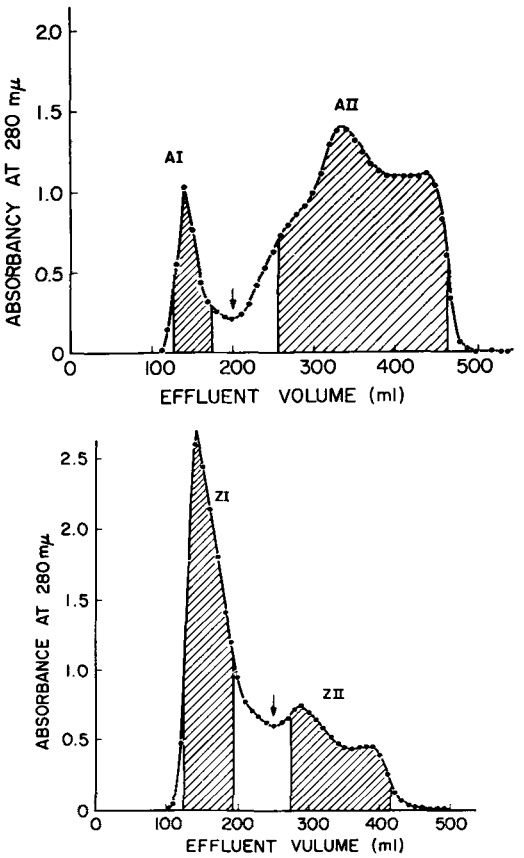

FIG. 1. Elution profiles obtained by exclusion chromatography of acid casein (A) and Casein Z (Z). 400 mg casein, 2.5- by 95-cm column of G-150, 25 C, 0.005 M tris-citrate buffer, pH 8.6, containing 6 M urea. Arrows indicate demarcation between peaks for area calculations; hatched areas indicate pooled samples.

ably retarded and was clearly separated from Fraction I even when the protein giving rise to Fraction I predominated. Based on relative peak areas, uncorrected for differences in absorbance, Fraction I varied from 13% of the total for acid casein to about 70% for the κ-casein-rich Casein Z. Actual yields of freeze-dried materials from the eluent fractions designated in Figure 1, and following dialysis, concentration, and freeze-drying procedures which involved some loss of material, were 30 to 35 mg (8-9%) from 400 mg of acid casein and about 200 mg from Casein Z. The total yield of freeze-dried casein from the column amounted to approximately 90% of the original 400-mg sample.

Polyacrylamide gel electrophoresis patterns of Fractions I and II, and these same fractions after treatment with rennin (Fig. 2), indicate that Fraction I was always a κ-casein of high purity, and that Fraction II contained very little κ-casein. In some tests (e.g., Fig. 2, ZI + R) some material with the mobility of κ-casein remained after rennin treatment, but the coincidence of these zones with major κ-casein zones suggests an incomplete rennin action rather than the presence of contaminating protein.

Sialic acid determinations (Table 1) also indicate that Fractions I were κ-caseins of high purity and relatively constant composition, and that Fractions II contained little κ-casein. The constant composition of the κ-casein, regardless of the diverse casein preparations placed on the column, is also apparent from a comparison of the electrophoretic patterns (Fig. 3). These patterns appear to differ only in the intensity of the minor bands moving towards the cathode. These occurred only in κ-caseins separated from κ-casein-rich caseins, and probably indicate the presence of *para*-κ-casein-like components formed by degradation of κ-casein during the enrichment procedures. However, κ-caseins prepared by gel filtration at 4 C were not detectably different from those prepared at room temperature.

The 2.5- by 95-cm column of Sephadex G-150 was capable of separating κ-casein from 1 g or more of sample. In one case, five successive 1-g samples of soluble casein (0.50% sialic acid) were processed through a column, without repacking, to obtain 690 mg of pure κ-casein. Larger amounts of pure κ-casein were obtained by gel filtration of 1-g aliquots of a crude κ-casein (similar to Casein Z).

The stabilizing power of Sephadex-purified κ-casein was equal to that of the best κ-caseins

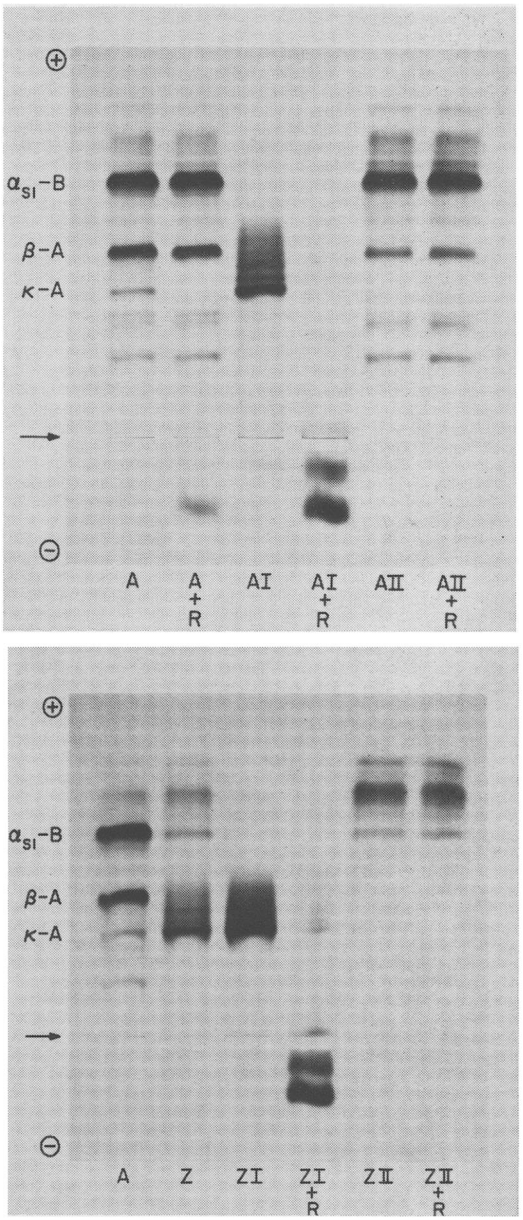

FIG. 2. Polyacrylamide gel electrophoresis patterns for acid casein (A), Casein Z (Z), and fractions (Fig. 1) of these samples before and after treatment with rennin. Arrow indicates origin. Rennin treated samples are designated as +R.

prepared by other methods. Application of Zittle's procedure (19) indicated that these κ-caseins were capable of stabilizing α_s-casein at a ratio of 0.14 κ- to 1.0 α_s-casein.

Discussion

The gel filtration method for preparation of κ-casein described in this paper provides a

TABLE 1
Sialic acid content[a] of whole caseins, Caseins S, H, and Z, and the fractions obtained by gel filtration on Sephadex G-150

Casein	Sialic acid (%)		
		Sephadex fraction[b]	
	Original	I	II
Acid casein	0.49	2.44	0.13
First-cycle soluble	0.50	2.42	0.12
S	1.21	2.80	0.31
H	1.48	2.51	0.61
Z	2.07	2.25	0.57

[a] Moisture-free basis.
[b] Hatched portion in the elution diagrams.

means of isolating pure κ-casein from whole casein in a single step, and is thus less involved than the available methods (2, 4, 7, 12, 20). It is particularly suitable for preparing pure κ-caseins, in good yield, from small amounts of whole casein. Moreover, if large amounts of κ-casein are to be isolated, gel filtration is an effective means of purifying κ-casein-rich materials such as Casein S or Z.

As a purification procedure, gel filtration is preferable to either DEAE-cellulose chromatography (1, 2, 7) or ethanol precipitation (4, 7, 20). The separation of unreduced κ-casein from other casein materials is more complete with Sephadex G-150 than with DEAE cellulose columns, and the gel column can be used repeatedly without repacking. It has been suggested (1) that urea splits κ-casein, but the absence of the *para*-κ-casein-like components when acid casein or soluble casein was used as the starting material, and the high and relatively uniform sialic acid content of the κ-caseins obtained, suggest that no degradation of κ-casein, detectable by the methods used, occurs during Sephadex gel filtration in 6 M urea. The use of ethanol has been reported (20) to give, sometimes, κ-caseins with reduced ability to stabilize α_s-casein; Sephadex purified κ-casein has excellent stabilizing properties.

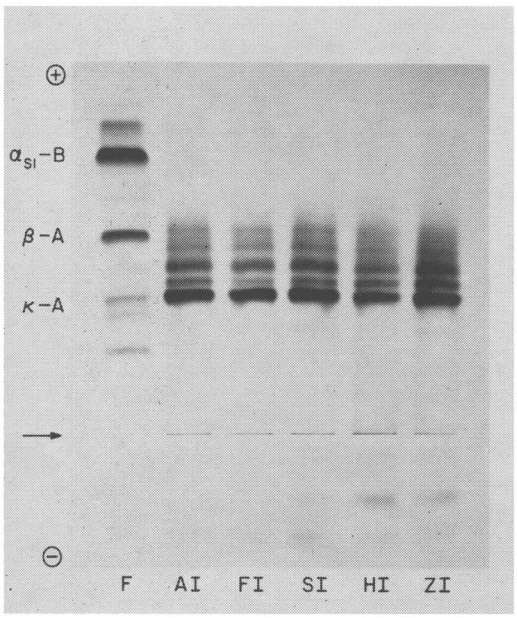

FIG. 3. Polyacrylamide gel electrophoresis patterns of first-cycle soluble casein (F) and of the first fraction obtained from acid casein (AI), first-cycle soluble casein (FI), Casein S (SI), Casein H (HI), and Casein Z (ZI). Arrow indicates origin.

Acknowledgment

The authors thank Dr. Dyson Rose for helpful criticism and J. R. Marier for the sialic acid determinations.

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