Fractionation of Caseins Directly from Skimmilk by Gel Chromatography. 1. Elution with Sodium Dodecylsulfate

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Abstract
κ-Casein was isolated directly from skimmilk without separating acid casein for application to a Sephadex G-100 column by eluting with 1.5 mM sodium dodecylsulfate at 4°C. The separated κ-casein contained bound dodecylsulfate and revealed decreased stabilizing ability, approximately 70%, against αs1-casein in the presence of Ca++. Partial elimination of bound dodecylsulfate by dialysis in urea and 2-mercaptoethanol restored most of the stabilizing ability of κ-casein. However, no simple method for removing the dodecylsulfate completely was found except acetone extraction which aggregated κ-casein with decreased stabilizing ability.

Electrophoretically pure αs-casein was separated from acid casein on Sephadex G-100 by eluting with 0.5 mM sodium dodecylsulfate. κ-Casein stabilized this αs-casein against Ca++ to the same extent as the αs1-casein prepared by the method of Zittle and Custer.

Introduction
A number of methods have been proposed for fractionation of the casein complex in milk. For separation of κ-casein, methods by Zittle and Custer (13) or by McKenzie and Wake (8) have been widely utilized. Zittle's method is relatively simple and produces nearly pure κ-casein with a high yield; however, the important procedural step is a strong acid treatment at a high urea concentration. McKenzie's method is relatively mild although more complicated, yielding less κ-casein. Yaguchi, Davies, and Kim (11) introduced gel chromatography for separating κ-casein from acid casein; however, a high concentration of urea was necessary for elution. Cheeseman (3) eluted a κ-casein rich fraction from acid casein on a Sephadex G-200 column with a sodium dodecylsulfate (SDS)-ethylenediaminetetraacetic acid (EDTA) eluent.

We searched for a mild procedure to separate κ-casein which would be acceptable for use in food processing. Cheeseman's procedure was carefully studied and we found that κ-casein could be separated directly from skimmilk, after modifying the eluent. Sephadex G-100 was selected since elution is faster than with G-200. αs-casein was separated acceptably pure from acid casein after further dilution of SDS for elution.

This paper reports our analyses of the separation of αs- and κ-caseins by gel chromatography on Sephadex G-100 eluted with SDS.

Materials and Methods
Caseins. κ-Casein A and αs1-casein B were prepared by the method of Zittle and Custer (13) from milk of a homozygous cow. κ-Casein A was also separated by the method of McKenzie and Wake (8). All chemicals were of reagent grade, except SDS which was of USP grade from Fisher Scientific Company.

Gel chromatography. Sephadex G-100, G-150, and blue dextran were purchased from Pharmacia Fine Chemicals, Inc. Upward flow system was employed for elution at 4°C. Sodium dodecylsulfate at 1.5 and 0.5 mM eluted κ- and αs-caseins after adjusting to pH 8.5 to 9.0. For separation of κ-casein, 5 ml of 5% Na2EDTA were added to 10 ml of skimmilk, pH adjusted to 8.5 with NaOH, centrifuged to eliminate turbidity due to residual lipids, and then applied to the column. To increase the yield of κ-casein, caseins precipitated from the last half of the κ-casein peak of a previous run may be added to the skimmilk. Skimmilk was stored at −20°C after adding EDTA and adjusting pH to 8.5, or after freeze-drying without deteriorating the resolution of κ-casein for six months. For separation of αs-casein, acid casein was applied after dissolution at pH 7.5. The effluent was monitored by absorbance at 280 nm with an Ultraviolet Analyzer UA-2, (Instrumentation Specialties Company, Inc., Lincoln, Nebraska) or analyzed sequen-
tially in test tubes from fraction collectors with a SP 800 Spectrophotometer (Pye Unicam Ltd., Cambridge, England). To ensure purest $\kappa$-casein, the first half of the first peak of 1.5 mM SDS elution was collected. The last half of the second peak of 0.5 mM SDS elution was collected for $\alpha$-casein. Fractions from the collector were combined, dialyzed against running tap water for 24 hr, and then caseins were precipitated by adding molar ammonium sulfate at pH 3.0. Dodecylsulfate bound to casein was removed by extracting with cold acetone (4).

Polyacrylamide gel electrophoresis. Aschaf-fenburg's procedure (1) was followed. Prior to sample application, gel was conditioned overnight under the same conditions as for electro-phoresis.

Sodium dodecylsulfate. Dodecylsulfate bound to casein was analyzed colorimetrically (7).

Stabilization of $\alpha_1$-casein with $\kappa$-casein. Zittle’s method (12) determined the stabilizing ability of $\kappa$-casein against standard $\alpha_1$-casein. Stability of $\alpha_1$-casein in the presence of standard $\kappa$-casein was estimated by the method of Beveridge and Nakai (2).

Ultracentrifugation. A Spinco L2-65B ultracentrifuge with schlieren optics was used. Caseins were dissolved in imidazole-KCl, pH 6.8, $\Gamma/2$ of .08 unless stated otherwise.

Results and Discussion

Elution of whole casein with sodium dodecylsulfate for separation of $\kappa$-casein. In preliminary experiments acid whole casein was used to find optimum conditions for separating $\kappa$-casein by gel chromatography. Concentration of SDS was varied for elution from a Sephadex G-100 column at 25 C (Fig. 1). No distinct peak was observed at 0.1 mM and absorbance at 280 nm was still high and far beyond the total volume ($V_t$) position. When SDS was increased to 0.2 mM two peaks appeared on the elution profile. As the concentration increased the second peak became broader and approached the first peak which contained more $\beta$- and $\alpha$-caseins (Fig. 2). Gel electropherograms (Fig. 2) indicated that $\kappa$-casein eluted at the void volume ($V_o$) at all concentrations above 0.2 mM and the best resolution was with 1.0 mM SDS at 25 C. $\alpha$-Casein was recovered from the last half of the second peak with 0.5 mM SDS.

The hydrocarbon tail of the sodium dodecylsulfate penetrates the apolar regions of proteins and increases electrostatic repulsion between the charges of bound species, resulting

![Graph](image-url)
in unfolding of protein conformation (10). Cheeseman and Jeffcoat (4) reported changes in size of the protein-SDS conjugates. At 10 to 20 moles of SDS per mole of whole casein, most of the \( \kappa \)-casein dissociated to an 11S component whereas all \( \alpha_{21} \)-casein and most of the \( \beta \)-casein dissociated to 3S components. This explains separation of \( \kappa \)-casein from other caseins on SDS-Sephadex columns.

Delayed elution of \( \kappa \)- and \( \alpha_{2} \)-caseins was observed especially at low SDS concentrations (Fig. 1). Herries, Bishop, and Richards (6) stated that SDS monomers adsorbed considerably to the gel matrix of Sephadex G-25. However, SDS at concentrations above critical micelle concentration, was excluded from the interior of gel particles and reacted qualitatively as expected for a reversible polymerizing system. Therefore, it is possible that SDS at low concentration binds gel as well as casein, causing a delay of elution. Void and total volumes calibrated with blue dextran and sucrose remained the same when the column was eluted with a phosphate buffer, pH 7.0.

Effects of buffering the SDS eluents were determined by adding 5.0 mM phosphate to 1.0 mM SDS and adjusting pH to 6.0, 7.0, and 8.0. Best resolution was at pH 6.0, however resolution of peaks generally deteriorated by buffering. The eluent of Cheeseman (3), 0.05 M Tris-HCl, pH 7.6 containing 1.0 mM EDTA and 20 mM SDS did not improve the resolution. Increasing ionic strength and pH produced only adverse effects. Sometimes crystalline dodecylsulfate clogged the column when the pH was not adjusted. Sodium hydroxide was added to SDS solutions to increase pH to 8.5 to prevent formation of SDS micelles.

Dodecylsulfate bound to \( \kappa \)-casein, 39 moles SDS per 20,000 g casein, was removed by Cheeseman and Jeffcoat's (4) method. However, as this method was tedious, simplifications were attempted. Dialysis against 4M urea containing 2-mercaptoethanol eliminated about half of the bound SDS and restored approximately 70% of the stabilizing ability to \( \kappa \)-casein against \( \alpha_{21} \)-casein; however, a large amount of urea was required for dialysis. Acetone extraction at 25 C produced some insoluble aggregates and excessive extraction at either 0 or 25 C had to be avoided as a para-\( \kappa \)-casein like band appeared on gel elec-

![Figure 2](image-url)
tropherograms resulting in decreased stabilizing ability of \(\kappa\)-casein against Ca-\(\alpha_\text{sl}\)-caseinate precipitation. No SDS was detected in \(\kappa\)-casein after the acetone extractions. However, none of the simpler methods for eliminating SDS from \(\kappa\)-casein separated with SDS were effective. After elimination of SDS, \(\kappa\)-casein was fairly pure electrophoretically (Fig. 3). Decreased stabilizing ability of \(\kappa\)-casein was not improved by extraction with cold acetone and aggregates sedimented rapidly; furthermore a small 16.7S peak was observed in the schlieren pattern by ultracentrifugation.

**Elution of skim milk for separation of \(\kappa\)-casein.** A column, 3.8 by 140 cm, was built in a cold room at 4°C to retard bacterial growth during repeated elution. Sodium dodecyl sulfate was increased to 1.5 mM at 4°C. A slight contamination of \(\kappa\)-casein with \(\alpha_s\) and \(\beta\)-caseins was observed by elution with 1.0 and 2.0 mM SDS. Thirty to 40 ml of skim milk were applied to the column for each run. Direct application of skim milk or of skim milk containing SDS to the column did not produce the sharp first peak for \(\kappa\)-casein separation. Complete dissociation of micelles in skim milk with EDTA was necessary before application to the column.

The \(\kappa\)-casein peak from skim milk stored frozen at -20°C for 10 weeks revealed a band at the \(\beta\)-lactoglobulin position in addition to the typical \(\kappa\)-casein bands. This could be avoided by storing skim milk at -20°C after freeze-drying or after addition of EDTA to skim milk. Commercial skim milk powder sometimes showed this band in the \(\kappa\)-casein fraction separated on the SDS column. Possibly \(\kappa\)-casein interacted with \(\beta\)-lactoglobulin, especially in the presence of Ca\(^{++}\) and eluted with \(\kappa\)-casein. An attempt to dissociate \(\beta\)-lactoglobulin from \(\kappa\)-casein by acid precipitation in the presence of 2-mercaptoethanol failed.

**Elution of whole casein for separation of \(\alpha_s\)-casein.** Acid whole casein for separation of \(\alpha_s\)-casein was applied to Sephadex G-100 and eluted with 0.5 mM SDS at 4°C. The separation of \(\alpha_s\)-casein from whey proteins in skim milk was incomplete. The last half of the second peak (Fraction D in Fig. 1 at 0.5 mM) was collected. The separated \(\alpha_s\)-casein was reasonably pure by gel electrophoresis (Fig. 2). A single peak was observed in the schlieren pattern from ultracentrifugation and the sedimentation coefficient was 1.3 S in 0.1 M glycine, pH 11 for 1.0% casein.

Stability of \(\alpha_{\text{sl}}\)-casein with increased \(\kappa\)-casein in the presence of Ca\(^{++}\) (2) was determined. There was no significant difference between \(\alpha_{\text{sl}}\)-caseins prepared by the SDS-gel chromatography and by Zittle’s (13) method. \(\alpha_s\)-Casein separated by this method contained 0.6 mole of bound SDS per 27,000 g casein which was low compared to \(\kappa\)-casein and SDS was completely removed by washing twice with cold acetone. The yield of purified \(\alpha_s\)-casein was approximately 17% of applied acid casein.

Attempts have been made to simplify procedures or to seek milder ones for the preparation of \(\kappa\)-casein from acid casein or skim milk. Isolation of \(\kappa\)-casein from whole casein by addition of CaCl\(_2\) is mild, however, in general purity as well as the yield is lower than with urea and sulfuric acid. An additional alcohol fractionation (8) is usually necessary to improve the purity of preparations. We replaced urea with acetic acid and SDS in Zittle’s method (13). Sulfuric acid was added to casein in 50% acetic acid to lower pH and diluted similar to Zittle and Custer’s (13) method or CaCl\(_2\) was added to casein in acetic acid to precipitate Ca-sensitive caseins. The purity and yield by these methods were similar to those by Zittle and Custer’s method with easier recovery of acetic acid by evaporation. Selective precipitation of \(\alpha_s\)- and \(\beta\)-caseins by adjusting pH or by adding CaCl\(_2\) in the presence of SDS was not as effective as urea or acetic acid as dis-

![Fig. 3. Gel electropherograms of \(\kappa\)-caseins prepared by different methods. W, whole casein; Z, Zittle and Custer’s method (13); PS and PA, eluted from Sephadex G-150 with a phosphate, pH 10.8 from skim milk and acid casein, respectively, as described in the following paper (9); S, eluted from G-100 with 1.5 mM sodium dodecyl sulfate at 4°C from skim milk; M, McKenzie and Wake’s method (8).](image-url)
sociating agents although most of the β-casein was eliminated from whole casein.

Sodium dodecyl sulfate-gel chromatography is economical as indicated in the cost ratio of 83:1 per unit volume of eluents calculated for the same grade of reagents for the urea-Tris-citrate method (11) and the SDS method. A considerable simplification in fractionation of caseins may be accomplished if the application of the continuous gel filtration apparatus (5) is feasible.

References


