Fractionation of Caseins Directly from Skimmilk by Gel Chromatography. 2. Elution with Phosphate Buffers

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Abstract

κ-Casein was separated on Sephadex G-100 by eluting with 0.01 M borate, pH 10 at 25 C or 0.01 M phosphate, pH 11 at 4 C. Phosphate, 5 mM with 2 mM Na ethylenediaminetetraacetate, pH 10.8, was used for continuous fractionation at 4 C to retard bacterial growth in the gel. κ-Casein was collected from the first half of the first peak. The stabilizing ability of the κ-casein against Ca-κ-caseinate precipitation was slightly low possibly from contamination with minor members of the α_s-casein group. Electrophoretically pure β-casein was obtained from the first half of the second peak after elimination of contaminating α_s-casein by precipitation at pH 4.4 and 4 C. α_s-Casein was purified from the last half of the second peak by precipitating with 0.1 M CaCl₂. The phosphate method was superior to the sodium dodecylsulfate method because of faster elution, being more reproducible, and without extraction to remove bound dodecylsulfate. α_s-, β-, κ-Caseins were fractionated directly from skimmilk, however, the yield of κ-casein was approximately half of that by the sodium dodecylsulfate method.

Introduction

Nakanishi and Ito (6) reported that excessive mixing or agitation during purification of κ-casein with alcohol and ammonium acetate by Zittle and Custer's (11) method resulted in gelation of κ-casein probably due to oxidation of sulfhydryl groups, and that repeated purification increased degradation of casein and produced bands similar to para-κ-casein on a gel electropherogram. Methods of fractionating casein micelles in milk have been investigated and we found that elution with dilute phosphate buffer, pH 10.8 at 4 C isolated κ-casein directly from skimmilk. α_s- and β-Caseins were also separated simultaneously from later stages of the elution by additional steps for further purification.

This paper deals with this new method for direct separation of caseins from skimmilk by gel chromatography.

Materials and Methods

Gel chromatography. A phosphate buffer, 5 mM, pH 10.8, containing 2 mM sodium ethylenediaminetetraacetate (Na₂EDTA) was the eluent at 4 C. Two drops (0.06 ml) of HCl were added to each test tube in a fraction collector, prior to elution, to neutralize 12 ml of effluent immediately after flowing from a Sephadex column, 3.8 by 140 cm. As seen in Figure 1 skimmilk was applied to the column after treatment as described in the preceding paper (4). Fractions of κ-casein collected from the first half of the first peak, earlier than that by SDS elution, were combined and the casein precipitated by adjusting to pH 4.5. For α_s-casein the last half of the second peak before a shoulder appeared in the descending half, (probably from whey proteins) was combined, the pH was adjusted to 4.2 at 4 C, and the precipitate after centrifugation at 4 C was dissolved at pH 7.5 and 25 C. The casein was reprecipitated by adding 0.1 M CaCl₂, dissolved by adding 20% potassium oxalate to pH 7.0 and dialyzing the supernatant after centrifugation. For β-casein the first half of the second peak was adjusted to pH 4.4 at 4 C and the supernatant after centrifugation at 4 C was warmed to 25 C and pH 4.6. β-Casein was then collected by centrifugation.
Fractionation of Caseins

Skimmilk

Add 1.7% EDTA and adjust to pH 8.5

Sephadex column

Elute with 5 mM phosphate-2 mM EDTA, pH 10.8

Division A

Adjust to pH 4.5 and centrifuge

Sediment (κ-casein)

Division B

Acidify to pH 4.4 and centrifuge at 4°C

Supernatant

Adjust to pH 4.6 at 25°C and centrifuge

Sediment (β-casein)

Division C

Acidify to pH 4.2 and centrifuge at 4°C

Sediment

Dissolve at pH 7.5 and 25°C, add 0.1 M CaCl₂ and centrifuge

Supernatant

Dissolve in 20% K₂C₂O₄ and centrifuge

Supernatant

Dialyze

α₂-casein

Fig. 1. Fractionation of casein by Sephadex gel chromatography.

Sialic Acid. Determined by the method of Warren (10).


Results and Discussion

Elution of whole casein and skimmilk with phosphate buffers for separation of κ-casein. Preliminary tests for determining effects of pH and temperature of eluents on resolution were performed on a small Sephadex G-100 column with a water jacket for controlling temperature. Acid casein was applied to the column (Fig. 2). For separation of κ-casein at 25°C, the best resolution was obtained at pH 10 with 0.1 M borate. At 45°C and pH 10, size of the κ-casein peak decreased probably because of excessive dissociation. At 4°C, pH had to be increased to 11 for best resolution. At low temperatures or low pH caseins dissociate insufficiently, resulting in inadequate separation of κ-casein. Whereas too high temperatures and pH probably split disulfide linkages in κ-casein (5, 7) and degraded κ-casein moves to the lower molecular weight side admixing with the β- or α₂-fraction. Consequently, separation of κ-casein is inadequate. The best condition for the separation of κ-casein is inadequate.
casein was pH 10 at 25°C or pH 11 at 4°C. Most caseins in the first peak at the void volume (V₀) revealed fairly pure κ-casein bands on the gel electropherogram, except the one eluted at pH 9 and 4°C. The first peak disappeared completely by eluting with 0.1 or 0.2 M NaOH at 4°C because of complete dissociation of κ-casein from cleavage of disulfide groups. Neither 2 nor 4 M acetic acid clearly separated κ-casein. Most of the caseins were eluted at V₀ indicating incomplete dissociation. When 2-mercaptoethanol was added to acid casein before application to the pH 11 column at 4°C, the size of the κ-casein peak decreased considerably.

Mechanisms of separation of κ-casein from other caseins may be ascribed to molecular size differences among dissociated and unreduced κ, α₁, and β-caseins. In the presence of dissociating agents such as urea, sodium dodecylsulfate or weak bases, α₁ and β-caseins disaggregate to monomers of 20 to 27 x 10³ mol wt whereas κ-casein remains at a higher molecular weight of 90 to 125 x 10³ (7). However, when disulfide groups are cleaved with 2-mercaptoethanol or stronger bases, κ-casein is reduced to monomers of the same order of molecular weight as α₁ and β-casein monomers. Disulfide groups in κ-casein begin cleavage at above pH 10 at 25°C (5, 7), agreeing with gel chromatographic results. If all sulfur groups in κ-casein in skim milk were reduced, no κ-casein peak would appear at V₀ during gel chromatography on Sephadex G-100 in the presence of dissociating agents.
Although elution at pH 10 and 25°C yielded fairly pure κ-casein from acid casein (Fig. 2), elution conditions at pH 11 and 4°C were selected for skim milk to prevent possible protein denaturation. Several fractions were collected from the peak at Vo and analyzed by gel electrophoresis (Fig. 3). Division A contained almost pure κ-casein whereas αs1- and αs4-like caseins contaminated κ-casein in division B and as elution proceeded, β- and αs1-caseins also contaminated κ-casein. Thus only the first half of the Vo peak was collected for κ-casein. The last half was precipitated at pH 4.5 and reapplied to the column again with the next sample of skim milk.

Purification of αs- and β-caseins. β-Casein was purified by Warner's method (8) from the first half of the second peak (Fig. 1). This casein was electrophoretically pure (Fig. 4). αs-Casein was purified by adding CaCl₂, a slight modification of the method for separation of the fraction P by Wang and von Hippel (9). Electrophoresis indicated minor bands slower than αs1-band probably αs3- and αs4-caseins which were not removed completely (Fig. 4).

Comparison of the phosphate and the sodium dodecylsulfate methods for fractionating caseins. Purity of κ-casein separated by both elution methods was equally and reasonably high [Fig. 3 in (4)]. κ-Caseins separated from skim milk and from acid casein were of the same purity. However, the contamination in κ-casein fraction increased faster for the phosphate method than for the SDS method as fractionations advanced within the Vo peak region. Therefore, the earlier fraction of the first half, before reaching maximum absorbance, had to be collected by the phosphate method, as compared to the SDS method. This reduced the yield for the phosphate method to 70 mg compared to 200 to 220 mg for the SDS method from 100 ml of skim milk with Sephadex G-100. These yields are of the same order as those by McKenzie's (3) and Zittle's (11) methods. The yield for the phosphate method was improved to 100 mg with Sephadex G-150, pH 10.8 at 4°C. In general the purity of κ-casein can be improved by a slight adjustment of pH or the SDS concentration for the phosphate and the SDS methods.

The flow rate of elution was slower for the SDS method, approximately 80% of that for the phosphate method. Furthermore, delayed elution of the SDS method because of the increased elution volume of 30% over the phosphate method, further delayed elution rate. Altogether the rate of elution for the SDS method was 60% of that for the phosphate method. The flow rate continued decreasing for the SDS method even on the upward flow column and frequent stirring of the upper part of the column was necessary. Electrophoretically pure αs- and β-caseins were isolated from the same column by the phosphate method, applying skim milk without prior separation of acid casein (Fig. 4), whereas acid casein had to be applied for isolation of αs-casein by the SDS method. In addition, washing with acetone was required to liberate SDS bound to caseins which might result in denaturation of casein molecules.

The cost of eluents for the phosphate method was approximately the same as that for the SDS method. The reproducibility of elution with phosphate on the same column was so successful that 50 elutions were continued on an upward flow column without repacking.

Properties of κ-casein isolated by gel chromatography. The stabilizing ability of κ-caseins, separated by gel chromatography eluted with phosphate buffer, against Ca-αs1-caseinate precipitation was slightly low compared to Zittle's κ-casein (11). This difference of 10 to 15% may be explained by contamination of κ-casein with minor members of the αs-casein group. This corresponds to approximately 15% fewer disulfide groups estimated by the p-mercuribenzoate method (5) in κ-casein prepared by the phosphate method than Zittle's κ-casein. Alcohol purification (3) removed most of the contaminating αs1- and β-caseins but not αs3- and αs4-caseins.

Sialic acid in κ-caseins was 1.85, 1.65, 2.19,
and 1.89% for Zittle’s method, the phosphate method from acid casein, and from skim milk, and the SDS method from acid casein, respectively. Hill and Hansen (2) reported that sialic acid was reduced about 20 to 30% during acid precipitation, accounting for the higher content in $\kappa$-casein eluted directly from skim milk.

Sedimentation coefficient, $S_{20w}$ for 0.9% $\kappa$-casein preparations was measured. $\kappa$-Casein separated by the phosphate method had a significantly lower $S_{20w}$ of 13.0 compared to 16.0 and 14.5 for $\kappa$-caseins prepared by Zittle’s (11) and McKenzie’s (3) procedures. Interaction of $\kappa$-casein with $\alpha_1$-casein decreased the sedimentation coefficient, however, whether or not this contamination caused a lower coefficient was not established because of lack of sedimentation data on $\alpha_3$- and $\alpha_4$-caseins. The viscosity of a solution of Zittle’s $\kappa$-casein was higher than that prepared by gel chromatography which supports Nakanishi’s observation (6). Similarly, whether the alcohol purification was the only cause for gelation of Zittle and Custer’s $\kappa$-casein is still uncertain since $\kappa$-casein of McKenzie and Wake was purified similarly except that they used a lower concentration of alcohol.

$\kappa$-Casein separated by the SDS method contained 39 moles SDS bound to 20,000 g casein and indicated $S_{20w}$ of 10.7 accompanied by a small peak of a slow moving fraction of 3.9S. These values agree with the results of Cheeseman and Jeffcoat (1). After elimination of SDS with cold acetone $S_{20w}$ increased to 16.7. This result indicates a tendency towards aggregation of SDS-$\kappa$-casein after acetone extraction.

Gel chromatography may be useful for the direct fractionation of caseins from skim milk because of mild conditions and economy of procedure in addition to feasibility of automatic processing.

References