

DEAE-Cellulose-Urea Chromatography of Casein in the Presence of 2-Mercaptoethanol

2459

Abstract

κ -Casein, after reduction of the disulfide bond(s) by 2-mercaptoethanol, can be separated from α_{s1} - and β -casein variants, as well as from whole casein, by DEAE-cellulose-urea chromatography. When κ -casein is in the reduced form it elutes from DEAE-cellulose at 0.05 M NaCl. Use of a reduction step simplifies purification of the major casein components (α_{s1} , β) by chromatography.

Unlike α_{s1} - and β -caseins, κ -casein possesses peculiar behavior upon starch-gel electrophoresis; it migrates as a smeared, ill-defined zone (2). Many researchers have observed that κ -casein associates more strongly with β -caseins than with α_{s1} -caseins. Detection of small amounts of κ -casein in other casein components is difficult. (Although sialic acid content is a measurement of the presence of κ -casein, small amounts would be detected with difficulty, if at all.) Physical measurements offer little hope for its detection, because of interactions with α_{s1} - and β -caseins.

Renewed interest in κ -casein has been generated by the studies of Swaisgood and Brunner (6), who observed upon reduction of the disulfide of this protein that it consisted of at least two polypeptide chains of about 26,000 mol wt. A logical progression of observations resulted from this study; three laboratories working concurrently, but independently, of each other (3, 5, 10), reported that upon reduction of κ -casein, followed by zonal electrophoresis, two principal zones appear. Since variation in these zones (which occur either singly or in pairs) appeared among individual cows, genetic polymorphism was suspected. Unfortunately, however, since family studies or segregation data were not reported, the authors could not conclude that genetic variation was responsible for the polymorphism. Aschaffenburg (1) has since suggested that the variation in κ -casein has, indeed, a genetic basis; the

polymorphism (κ -A and κ -B) is universal among breeds studied.

This paper reports a method for removing κ -casein (regardless of genetic type) from preparations of α_{s1} -, β -, or whole casein. The method utilizes the fact that when κ -casein is reduced by 2-mercaptoethanol it elutes from DEAE-cellulose-urea columns at low ionic strengths (about 0.05 M NaCl). Without the incorporation of 2-mercaptoethanol, a portion of the κ -casein elutes in the β - and α_{s1} - regions, whereas some is absorbed to the exchanger and must be eluted with 0.2 N NaOH (4). Because α_{s1} - and β -caseins contain no disulfide, these proteins are unaffected and elute in their normal positions from the DEAE-cellulose columns (7, 8).

Materials and Methods

Sources and preparation of α_{s1} -, β -, and whole caseins. The sources and preparation of genetic variants of α_{s1} - and β -caseins from homozygous cows have been described (7, 8). Whole casein (typed α_{s1} -B, β -AC, and κ -B) was obtained from the milk of a Guernsey cow by isoelectric precipitation of casein at pH 4.6 and 20 C, followed by successive washings with water. The casein was dried by extraction of the water with successive washings of ethyl alcohol, acetone, and ether.

Preparation of samples for chromatography. One-half to one gram of lyophilized α_{s1} - and β -casein variants were dissolved in 50 ml of 0.01 M imidazole-HCl buffer at pH 7.0 and 3.3 M urea, and adjusted to pH 7.0 by addition either of 0.1 N NaOH or of 0.1 N HCl. One-half milliliter of 2-mercaptoethanol (ME) was added with stirring and the solution stored overnight at 4-5 C. Solvent-dried whole casein (1.5 g) was dissolved in 60 ml of the above buffer and adjusted to pH 7.0 with 1 N NaOH. To this solution 0.5 ml of ME was added.

Column chromatography. Chromatography of α_{s1} - and β -casein was performed essentially as previously described (7, 8). To insure maintenance of κ -casein in the reduced form for the duration of the chromatographic separation of

components, 0.1 ml ME was added to each reservoir (150 ml buffer) in the mixing chambers. The gradient elution of β -casein from a 2 by 20-cm DEAE-cellulose column was commenced at 0.00 M NaCl (0.00 to 0.21 M NaCl) instead of 0.05 M, to produce a salt gradient sufficiently slow to insure quantitative elution of κ -caseins. For α_{s1} -caseins, the salt gradient, 0.10 to 0.27 M NaCl (7) will, of course, elute all β -casein and κ -casein without interfering with the elution of α_{s1} - itself. Whole casein was eluted with a NaCl gradient of 0.00 to 0.30 M as employed heretofore (8). Following column chromatography, selected casein fractions were thoroughly dialyzed to remove urea and NaCl and were lyophilized.

Zonal electrophoresis. Polyacrylamide-gel electrophoresis (PAE) (7, 8) was employed to detect the presence of κ -casein and other casein contaminants in the β -casein preparations. Five to seven milligrams of each casein fraction was dissolved in 1 ml of 7.0 M urea-buffer, 0.05 ml of ME added, and the solution allowed to set overnight at 4-5 C. PAE runs were performed as usual at pH 9.1, tris, Na_2EDTA , H_3BO_3 buffer with 0.10 ml of ME added to 150-ml gel solution prior to pouring.

Results and Discussion

Until recently no adequate methods have been available for detection of small amounts of κ -casein in preparations of α_{s1} - and β -casein. Previously described methods of zonal electrophoresis in starch-gel (SGE) or PAE will not detect small amounts of κ -casein. Upon addition of ME to β -casein preparations (7), which appeared to be pure by PAE in the absence of ME, small but observable amounts (<5%) of κ -casein were observed. The presence of κ -casein as a contaminant of purified β -casein was confirmed by immunoelectrophoresis by Dr. R. Lyster, National Institute for Research in Dairying, Reading, England. The column chromatographic method described above, wherein ME is used to reduce κ -casein and maintain reduction throughout the course of column separation, is an adaption of a previously described method (4), further developed to remove all traces of κ -casein. Figures 1 and 2 demonstrate results of this method. The two patterns (Figure 2), represented as 0, are the crude β -caseins prior to column chromatography. Fraction I represents an enrichment of temperature-sensitive casein and other ill-defined components which elute from the columns at low NaCl concentrations. Fractions II and III correspond to the B and A variants

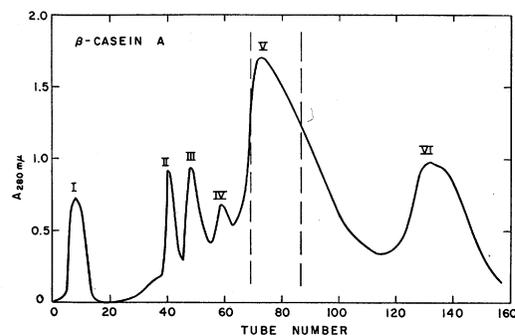


FIG. 1. Elution diagram (absorbance, A_{280} $m\mu$, versus tube number) of β -casein A from DEAE-cellulose, pH 7.0, imidazole-HCl-urea buffer in the presence of mercaptoethanol; 500-mg protein charge.

of κ -casein, respectively. Occurrence of the AB phenotype in this preparation of β -casein is fortuitous, but of interest is the good separation of the two; while the separation is good the two remain contaminated with each other. Fraction IV (Figures 1 and 2) is unidentified, but may constitute a carbohydrate-rich fraction of κ -casein. β -Casein (Fraction V in Figures 1 and 2) elutes in the characteristic asymmetric peak for this protein. Fraction VI constitutes a separation of SGE and PAE zones 1.00 and 1.04, tentatively characterized as α_{s2} (1.04) and α_{s3} (1.00) caseins (7). Rechromatography of Peak V (which amounts to 65% of the total protein eluted), β -casein, is shown in Figure 3. Only small amounts of contamination were observed; this fraction was now free of κ -casein by immunoelectrophoresis (R. Lyster). β -Caseins B and C have been similarly purified by the above method. Phosphorous analyses of β -caseins as chromatog-

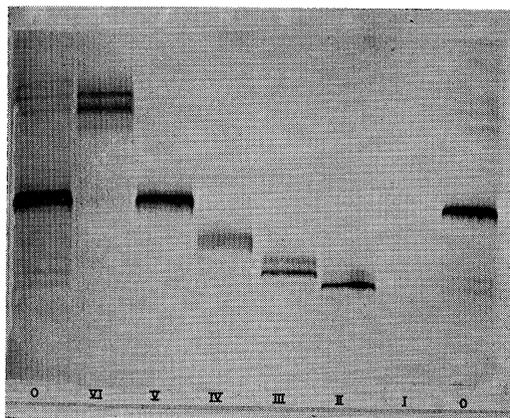


FIG. 2. Polyacrylamide-gel electrophoresis of fractions shown in Figure 1. See text for details.

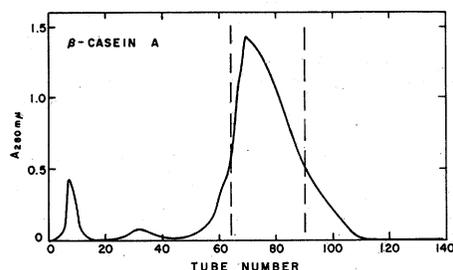


FIG. 3. Elution diagram of rechromatography of Peak V, Figure 1. Conditions same as in Figure 1.

raphed above were 0.58–0.60% for β -A and B and 0.50% for β -C. In free boundary electrophoresis, pH 8.6, $\Gamma/2 = 0.10$, veronal buffer, the mobility values for β -A, B, and C are -3.2 , -3.0 , and $-2.9 \times 10^{-5} \text{ cm}^2 \text{ v}^{-1} \text{ sec}^{-1}$, respectively, at 1% concentration.

Chromatography of the genetic variants of α_{s1} -caseins, in the presence of ME, offers no particular advantage in purification over the method described by Thompson and Kiddy (7). α_{s1} -A, B, and C were immunochemically pure. The α_{s1} -variants prepared by these authors were free of carbohydrates and cystine or cysteine. The chemical fractionation method used in the preparation of α_{s1} -caseins (calcium fractionation at low temperature and precipitation of contaminants from 50% ethyl alcohol by ammonium acetate) serves to remove κ -caseins, as well as other contaminating components.

DEAE-cellulose-urea chromatography of whole casein (typed α_{s1} -B, β -AC, and κ -B) in the presence of ME is shown in Figure 4. The initial peak (Fraction I) appears to contain a variety of casein components (Figure 5, I), the most concentrated of which corresponds in behavior to temperature-sensitive casein. Fraction II (Figures 4 and 5) corresponds to γ -casein (compared with authentic γ -casein, unpublished) and has a higher mobility than κ -casein B (Fraction III). The most apparent reason for the greater mobility of γ -casein (lower retention time on DEAE-cellulose), as compared with κ -casein B, is the molecular sieving effect afforded by acrylamide gels. Figure 5, Fraction IV, corresponds to the casein component (IV) seen in Figure 1. Separation of β -casein C from β -casein A is clearly seen in Figures 4 and 5 (Fraction V and VI, respectively). β -casein C prepared by this method is free of β -A and other visible contaminants whereas β -A is contaminated with β -C. Better separation of β -A from β -C is accomplished by

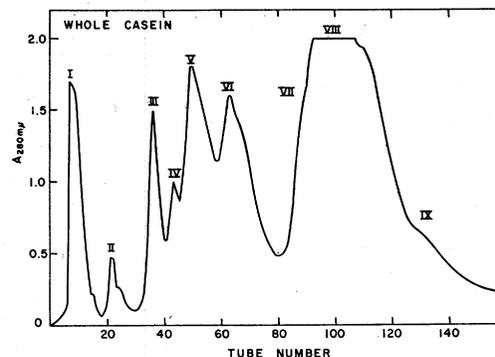


FIG. 4. Elution diagram of whole casein typed α_{s1} -B, β -AC, κ -B from DEAE-cellulose-urea in the presence of mercaptoethanol.

decreasing the quantity of total casein applied to the column. Attempts to separate β -casein A from β -casein B have met with failure. However, the abundance of β -casein B in the milks of Jersey cattle makes its separation from either A or C unnecessary. Fraction VII in Figures 4 and 5 represents an enrichment of zones 1.00 and 1.04, whereas Fraction VIII represents α_{s1} -B, which is only slightly contaminated. No casein components were eluted from the column when 0.20 N NaOH was applied to remove possibly adsorbed material, either with whole casein, β -, or α_{s1} -caseins. Use of ME, then, insures quantitative elution of all casein components with NaCl.

Finally, when ME is incorporated in PAE, two observations are made. First, the component referred to as 0.86 (9) on starch-gel electrophoresis disappears; the inference is that it contains disulfide. Secondly, when whole milk is typed for α_{s1} -, β -, and κ -caseins in the presence of ME by PAE, β -lactoglobulin B migrates in proximity to β -casein B, making an accurate phenotyping of β -casein difficult when either β -lactoglobulin AB or BB is present in the milk. A new method, thin starch-gel electro-

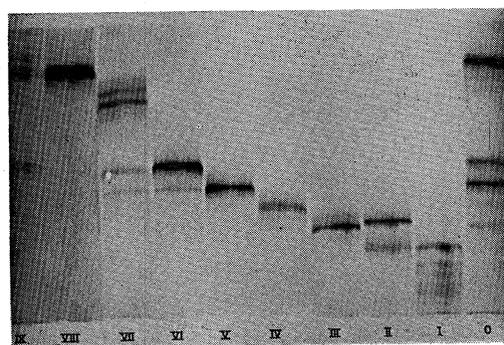


FIG. 5. Polyacrylamide-gel electrophoresis of fractions shown in Figure 4. See text for details.

phoresis in the presence of ME, devised by Aschaffenburg and Thymann (1), eliminates this problem, as does the method of Wake and Baldwin (9) when ME is introduced into the gel.

References

- (1) Aschaffenburg, R., and Thymann, M. 1965. Simultaneous Phenotyping Procedure for the Principal Proteins of Cow's Milk. *J. Dairy Sci.*, 48: 1524.
- (2) McKenzie, H. A., and Wake, R. G. 1961. An Improved Method for the Isolation of κ -Casein. *Biochem. et Biophys. Acta*, 47: 240.
- (3) Neelin, J. M. 1964. Variants of κ -Casein Revealed by Improved Starch Gel Electrophoresis. *J. Dairy Sci.*, 47: 506.
- (4) Ribadeau Dumas, B. 1961. Fractionnement de la Caséine par Chromatographie sur Colonne de Diéthylaminoéthyl-Cellulose en Milieu Uéré. *Biochem. et Biophys. Acta*, 54: 400.
- (5) Schmidt, D. G. 1964. Starch-gel Electrophoresis of κ -Casein. *Biochim. et Biophys. Acta*, 90: 411.
- (6) Swaisgood, H. E., and Brunner, J. R. 1963. Characteristics of Kappa-Casein in the Presence of Various Dissociating Agents. *Biochem. Biophys. Research Commun.*, 12: 148.
- (7) Thompson, M. P., and Kiddy, C. A. 1964. Genetic Polymorphism in Caseins of Cow's Milk. III. Isolation and Properties of α_{s1} -Caseins A, B, and C. *J. Dairy Sci.*, 47: 626.
- (8) Thompson, M. P., and Pepper, L. 1964. Genetic Polymorphism in Caseins of Cow's Milk. IV. Isolation and Properties of β -Caseins, A, B, and C. *J. Dairy Sci.*, 47: 633.
- (9) Wake, R. G., and Baldwin, R. L. 1961. Analysis of Casein Fractions by Zone Electrophoresis in Concentrated Urea. *Biochim. et Biophys. Acta*, 47: 225.
- (10) Woychik, J. H. 1964. Polymorphism in κ -Casein of Cow's Milk. *Biochem. Biophys. Research Commun.*, 6: 267.