RESEARCH PAPERS

STARCH GEL ELECTROPHORESIS OF VARIOUS FRACTIONS OF CASEIN

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SUMMARY

Preparations of α-, β-, γ-, λ-, and κ-caseins were compared by electrophoresis in starch gel and in free solution. Acid casein separated into at least 17 zones. All fractions of casein contained numerous proteins, but the preparatory procedures brought about considerable enrichment of certain zones while weakening or eliminating others, and a tentative identification of specific zones with each casein fraction was, therefore, possible. α_s-Casein appeared to be contaminated with traces of β-, κ-, and γ-casein, but the main contaminants were unidentified fractions travelling between α_s- and β-casein. β-Casein was contaminated with traces of γ- and κ-casein, and with the unidentified fractions observed in α_s-casein. Our preparations of γ-casein were heavily contaminated with κ- and β-casein, and contained lesser amounts of α_s-casein and of unidentified fractions. Identification of the κ-casein zone was confirmed by zone electrophoresis from urea solution into urea-free gel, whereupon the κ-casein formed a characteristic precipitation zone, and by electrophoresis in gels containing calcium. Comparison of three κ-casein preparations indicated differences among them, both in stabilizing power and in electrophoretic properties. The preparation of κ-casein with the least contamination from other casein components appeared to be partially denatured.

Interest in the calcium-insensitive, rennet-sensitive fraction of casein that von Hippel and Waugh (20) designated as κ-casein has led to the development of a variety of methods for the preparation of this or a similar fraction of casein (3, 6, 7, 9, 10, 12, 14, 15, 18, 21). The κ-caseins prepared in our laboratories by some of these methods differed in their ability to stabilize micelles of calcium-sensitive casein.

Zone electrophoresis in starch gel (SGE) has been an aid in assessing the heterogeneity and following the fractionation of numerous proteins (2, 16), and a preliminary publication indicated that the technique could be adapted to a study of casein fractions (22). Studies on several fractions of casein (α-, β-, γ-, λ-, and three κ-casein preparations) were, therefore, undertaken by means of electrophoresis in free solution and in starch gel, and representative results are presented herewith. The starch gel electrophoresis (SGE) procedure developed differs in several respects from that of Wake and Baldwin (23).

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MATERIALS AND METHODS

All caseins were prepared from fresh, bulk herd milk obtained from the Central Experimental Farm, Ottawa.[1]

Acid casein was prepared by isoelectric precipitation with hydrochloric acid, and thoroughly washed by repeated resuspensions in water.

α-Casein complex (the mixture or complex of α_s-, κ-, and λ-caseins), β-casein, and γ-casein were prepared by urea fractionation (5). Calcium-sensitive α-casein [α_s of (24), α_R of (7), α_1 of (11)] was prepared from α-casein complex, as outlined by Morr (12). λ-Casein was prepared by the method of Swaisgood and Brunner (19).

κ-Casein I was prepared by the constant pH method (dissolution of α-casein complex at

[1] Thanks are extended to staff of the Canada Department of Agriculture for the milk used in these tests.

pH 7.0, precipitation of α_s-casein with 0.2 M calcium chloride, dialysis of supernatant, and precipitation of κ-casein at pH 4.7) of Morr (12), except that the suggested extraction with chloroform was omitted. κ-Casein II was prepared by the method of Swaisgood and Brunner (18). In this method, α-casein complex (urea preparation) is dissolved in urea solution, α_s-casein precipitated with trichloroacetic acid and, after dialysis and concentration, with 0.25 M calcium chloride; residual traces of β-casein removed by centrifugation at 35 C and κ-casein precipitated at pH 4.4. κ-Casein III was prepared by the method of McKenzie and Wake (9).

All casein preparations were freeze-dried and stored at 4 C. Tests on each preparation were usually completed within 2 wk.

The micelle stabilizing power of κ-casein preparations was determined by preparing a solution of 0.48 g calcium-sensitive casein (α_s) and 0.12 g κ-casein in 16 ml of saturated calcium hydroxide solution and adjusting to pH 12.0 with 1 N sodium hydroxide. This solution was then neutralized to pH 6.5 by slowly adding 1 N hydrochloric acid from a pipette with the tip immersed in the solution, while stirring vigorously with a magnetic stirrer. An aliquot of the resulting suspension was centrifuged at 580 × g (at tip) for 10 min, the volume of sediment noted, and the supernatant decanted and analyzed for residual nitrogen (micro-Kjeldahl). Stabilizing power is expressed as the percentage of nitrogen remaining in suspension.

Moving boundary electrophoresis was done in standard 11-ml cells with a Spinco Model H apparatus, at a bath temperature of 1 C. Buffers consisted of sodium veronal, pH 8.1-8.3, $\mu = 0.1$, and sodium cacodylate, pH 7.0-7.2, $\mu =$ ca. 0.04.

Both one- and two-dimensional SGE was performed in the vertical apparatus described by Smithies (17). Starch—hydrolyzed for gel electrophoresis was obtained from Connaught Medical Research Laboratories, Toronto. The concentration recommended for serum proteins was 12 g starch per 100 ml buffer, but 15 g per 100 ml was found more suitable for casein studies, especially in concentrated urea. Electrophoresis was usually carried on for 7 hr in a constant current density of 1.5 ma/cm^2, with initial field strength of 3 and 4.5 v/cm in absence and presence, respectively, of concentrated urea.

Development of SGE procedure. To develop the SGE procedure, extensive studies were conducted on solutions of acid casein. Samples of acid casein, dissolved by titration to pH 7, 8, 9, 10, and 11, were subjected to SGE in urea-free sodium veronal, borate, or glycinate buffers, pH 7.3 to 10.6, $\mu = 0.025$. The resultant patterns indicated incomplete dissociation of the micelles. Buffers containing 2.7 M or more urea were effective in disaggregating the protein, both in the sample solutions and in the gels. For all subsequent tests, solid urea was added to the hot starch solution before degassing; the concentrations quoted are not corrected for the volume occupied by starch. Addition of urea increased measured gel pH by 0.1 to 0.2 unit.

Buffers tested as gel media included acetate—pH 5.2 to 6.1, sodium cacodylate—pH 6.2 to 7.2, sodium phosphate—pH 6.0 to 7.4, sodium veronal—pH 6.6 to 8.6, and sodium borate—pH 10.4, all containing concentrated urea. All protein components were anionic above pH 5, but isoelectric aggregation interfered with migration below pH 5.6. Mobilities increased gradually with pH, but above pH 7.0 the separation of major zones diminished; therefore, sodium cacodylate, pH 7.0 to 7.2, was preferred for comparison of more mobile components, and sodium veronal, pH 8.2 to 8.4, for the slower-moving zones. For routine operation near pH 7.0, buffers containing 5.5 M urea were used whereas, above pH 8.0, the urea concentration was reduced to 4.8 M to retain gel consistency.

The electrode and bridge solutions consisted of 0.5 and 0.1 M sodium chloride, respectively. Bridge solutions consisting of 0.15 M sodium borate, pH 8.6, rather than 0.1 M sodium chloride, did not significantly alter the resolution with these gel-buffer systems.

Tests with varied ionic strengths of urea-containing buffers indicated that $\mu = 0.020$ was optimal; lower concentrations tended to distort zones, while higher concentrations caused spreading of the zones. Increasing the starch concentration above 15 g/100 ml of buffer also caused distortion of the zones.

Under the conditions used, vertical SGE gave sharper fronts and better resolution than obtained in the horizontal plane.

RESULTS

Resolution of acid casein. Single-dimension SGE in cacodylate-urea, pH 7.2, $\mu = 0.020$, resolved acid casein into ten zones, some of

which were not discretely separated (Figure 1-A). Some of the more concentrated components (Zones 6-7 and 11-12) were better resolved in more dilute solutions, but the weaker zones disappeared upon dilution. Two-dimensional SGE (Figure 1-B) in veronal-urea, fol-

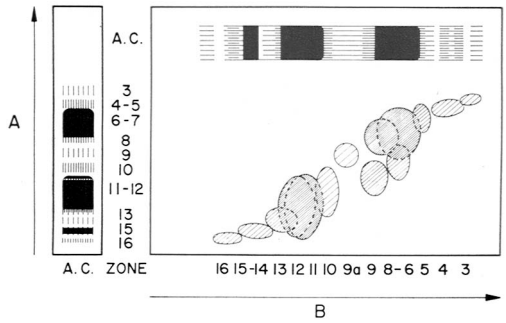

FIG. 1. Diagram of two-dimensional starch gel electrophoresis pattern of acid casein. One-dimensional controls of acid casein indicated by A.C. Relative intensity of spots indicated by shading. Gel media: A (first dimension)—5.5 M urea, sodium cacodylate, μ 0.020, pH 7.2. B (second dimension and its control)—5.5 M urea, sodium borate, μ 0.020, pH 10.4.

lowed by borate-urea, pH 10.4, or vice versa, confirmed the heterogeneity of these zones both by the asymmetry and by the irregular depth of staining of the zones in the second dimension. Including Zone 17, which was occasionally observed at the origin, acid casein thus was resolved into at least 17 components under these conditions.

Comparison of casein fractions. Single-dimension SGE patterns for various casein fractions in cacodylate-urea and veronal-urea buffer are shown in Figures 2 and 3, respectively. All of the fractions were resolved into several zones, but the preparative procedures have, in each instance, greatly enriched one or a few zones while diminishing or eliminating others. Both the α-casein complex and α_s-casein preparations were virtually free of Zone 12 protein, while the β-casein fraction contained a large amount of protein in Zone 12 and adjacent area. β-Casein, on the other hand, contained very little protein in the region of Zones 5 to 8. The γ-casein fraction contained considerable protein in the region of Zones 12 to 15 (the zones of β- and κ-caseins), but was the only fraction enriched in the component of Zone 16. Zone 16 thus appears to be specific to γ-casein.

Moving boundary electrophoresis of these same fractions gave essentially single peaks for α-casein complex and β-casein, with 95 and

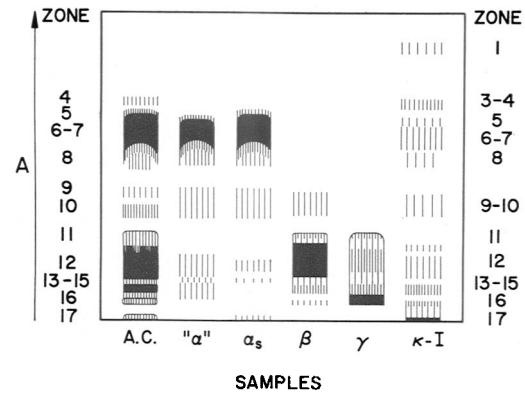

FIG. 2. Diagram of starch gel electrophoresis patterns of casein fractions at pH 7. One dimension in Medium A (See Figure 1). Samples: A.C., acid casein; α, α-casein complex; α_s, calcium-sensitive α-casein; β, β-casein; γ, γ-casein; κ-I, κ-casein by Method I (12).

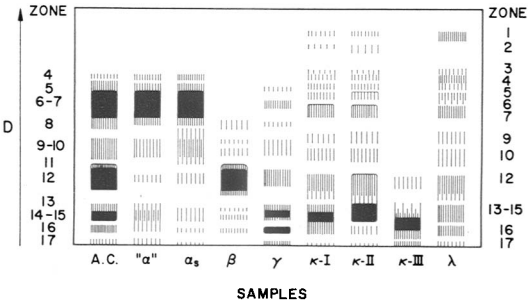

FIG. 3. Diagram of starch gel electrophoresis patterns of casein fractions at pH 8. One dimension in Medium C (4.7 M urea, sodium veronal, μ 0.020, pH 8.4). Samples: A.C., α, α_s, β, and γ as in Figure 2; κ-I, κ-II, and κ-III, κ-caseins prepared by Methods I (12), II (18), and III (9), respectively; λ, λ-casein fraction (19).

84%, respectively, of the total area under the descending pattern represented by the major peak. The γ-casein, on the other hand, appeared quite impure in moving boundary electrophoresis; based on area under the pattern, 10% of the preparation was γ-casein, 59% was β-casein, and 32% had a mobility close to that of α- or κ-casein.

Single-dimension SGE patterns for λ-casein and three κ-casein preparations in veronal-urea are shown along with other casein fractions in Figure 3. Again, all patterns indicated the presence of several proteins in each preparation. Relative to acid-casein, or to any of the other fractions, λ-casein preparations were enriched in the fast-moving (Zone 1) protein, although they also contained considerable amounts of most other zones. κ-Casein I con-

tained some material forming Zones 1 to 7, but was enriched in the proteins forming Zones 13 to 16, with some trailing back to the origin (Zone 17). κ-Casein II contained similar amounts of the faster-moving components, but was more enriched in Zones 13 to 15 and contained less Zone 16 component. κ-Casein III contained no components travelling ahead of Zone 12 and was enriched principally in Zones 15 to 17.

Moving boundary electrophoresis in either veronal or cacodylate buffer showed only one major component in κ-caseins I and II, with a mobility of −6.6 to −7.0 $cm^2\ sec^{-1}\ volts^{-1}$ (κ-casein I) and −7.8 to −8.0 (κ-casein II) in veronal, pH 8.2, $\mu = 0.1$. A similar result was obtained with a second preparation of κ-casein III (mobility −7.1 to −7.2), but the first preparation showed three incompletely separated boundaries with only 36% of the area being under the peak having a mobility close to −7.0 in veronal.

It has been reported elsewhere (13) that κ-casein dissolved in urea, or after first-dimension SGE in a urea-containing gel, forms a characteristic K-zone when it migrates electrophoretically into urea-free starch gel, either in the presence or absence of calcium. Such a zone was observed upon SGE in sodium veronal, pH 8.2 to 8.4, of whole acid casein, α-casein complex and γ-casein preparations, and in all preparations of κ-casein and of λ-casein (Figure 4). When κ-caseins I and II were dissolved in urea and subjected to SGE into urea-free gel, the faster-moving zones (contaminants) stained less intensely than when urea was present in the gel, presumably because these components were partially entrapped in the K-zone. With κ-casein III, no other zones were visible under these conditions. Formation of a K-zone by other casein fractions presumably indicates the presence of κ-casein as a contaminant.

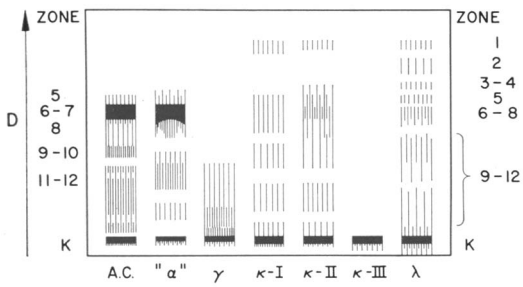

FIG. 4. Diagram of starch gel electrophoresis patterns in absence of urea. One dimension in Medium D (sodium veronal, μ 0.020, pH 8.3). Samples as in Figure 3.

The more mobile zones of the κ-caseins, and the major components of α-, β-, and acid-casein, were completely immobilized by 0.010 M calcium chloride in the electrolyte solutions, but migration of the κ-casein component itself was reduced only slightly. The fastest component of the λ-casein preparations also appeared to retain its mobility in the presence of 0.01 M calcium chloride.

Stabilization of casein micelles by κ-caseins. The ability of the various κ-casein preparations to stabilize micelles formed from 80% α_s- and 20% κ-casein varied widely (Table 1), but that of κ-caseins I and II appeared to be consistently greater than that of κ-casein III, except for the sample prepared from crude α-casein, which had been stored at room temperature for 2 yr. The stabilizing power of the λ-casein would appear to confirm the presence of considerable κ-casein (*cf.* Figure 3) in our preparation.

TABLE 1

Stabilization of casein micelles by κ-caseins

Casein preparation		Volume of precipitate formed	Nitrogen retained in suspension
		(ml/15 ml)	*(% of total)*
κ-casein I	1st preparation	<0.1	98
	2nd preparation	<0.1	100
κ-casein II	1st preparation	0.6	92
	2nd preparation	<0.1	100
	3rd preparation	<0.1	100
κ-casein III	1st preparation	1.1	37
	2nd preparation	1.0	71
κ-casein I, prepared from 2-yr-old α-casein complex		1.0	42
λ-casein		0.2	96

DISCUSSION

The SGE patterns obtained in these studies, including those in urea-free and in calcium-containing gels, permit the following tentative identifications of the 17 zones into which acid casein was resolved under these conditions:

Zone no.	Component
1	λ-casein [2]
2–4	Unknowns
5–8	Major components of calcium-sensitive α-casein (α_s-casein)
9–10	Unknowns, contaminants of both α_s- and β-casein
11–12	Major components of β-casein
13–15	κ-casein (especially Zone 14)
16	γ-casein (occasionally not distinct from Zone 17)
17	Unknown, probably a denatured fraction

None of the preparative methods studied yielded a product showing only a single zone in SGE.

The number of zones distinguished in these patterns is less than the total observed by Wake and Baldwin (23) in *tris*-citrate-urea buffer, pH 8.6. Possibly because Wake and Baldwin studied both lower and higher concentrations of casein than were used in our tests, they observed more zones in the vicinity of the major components and also observed five zones and a diffuse area in the region of Zones 13 to 15. The zone chosen by these authors as having a mobility of 1.00 appears to correspond to our Zone 8, giving a relative mobility of 1.7 for Zone 1 and of 0.4 for Zones 11-12 in our analyses in veronal-urea buffer. These values indicate a greater separation of the zones under our conditions.

Recently, attention has been drawn to the hazard of artefacts arising from carbamylation of proteins by ammonium cyanate in concentrated urea solutions (4). Since the same patterns were obtained with casein fractions dissolved in urea a few minutes before use, and those dissolved and held several days at 5 C, it is probable that such artefacts were not significant in our studies.

The similar mobility of α_s- and κ-casein in moving boundary electrophoresis indicates that these proteins have similar charge/mass ratios. The marked separation of these two components in SGE must, therefore, depend upon some other factor, such as the molecular sieving property (16) or differential adsorption. Molecular weights for completely dissociated α_s- and κ-caseins are quite similar [ca. 25,000 (8)], but molecular shape could also influence penetration through the gel. Zone 14 appears to be a single component, possibly with adjacent minor components. Although a series of zones was observed in this region by Wake and Baldwin (23), their presence in preparations treated with rennet suggests that most of them are not κ-caseins. However, a tendency for the component comprising Zone 14 to aggregate is suggested by its immobility on migration out of urea.

The method of Swaisgood and Brunner (18) appeared to yield the best κ-casein, even though many minor components were present as contaminants. The method of Morr (12) gave a similar product, except for more trailing from Zone 14 back towards the origin in SGE. Both of these preparations appeared to be quite reproducible, and both had a high stabilizing power for casein micelles. The method of McKenzie and Wake (9) yielded a κ-casein which was less contaminated with fast-moving components, but was more variable in behavior (both in SGE and moving boundary electrophoresis), and had a lower stabilizing power for casein micelles. Wake and Baldwin (23) also report a variable behavior of this κ-casein in SGE.

[2] Since α_s-, β-, γ-, and κ-caseins have been more or less clearly defined by their properties (electrophoretic mobility in free solution, calcium-sensitivity, etc.), the present authors believe it is correct to assign these names to one or more zones thought to be formed by proteins exhibiting these properties. On the other hand, the name λ-casein seems at present to be applied to an ill-defined fraction obtained by a certain preparatory procedure, and the propriety of assigning this name to the fast-moving, calcium-insensitive zone may be questioned.

ACKNOWLEDGMENT

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