

## PREPARATION OF $\beta$ - AND $\gamma$ -CASEINS BY COLUMN CHROMATOGRAPHY

M. L. GROVES, T. L. McMEEKIN, N. J. HIPPI AND W. G. GORDON  
*Eastern Regional Research Laboratory\*,  
Philadelphia, Pa. (U.S.A.)*

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### SUMMARY

A new method for preparing  $\beta$ - and  $\gamma$ -casein is described consisting of fractionation of that portion of casein which is soluble at pH 4 at 2° followed by chromatography on ion-exchange cellulose. The homogeneity of the preparations is evaluated by means of starch-gel electrophoresis in the presence of urea.

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### INTRODUCTION

A number of methods have been devised for separating the principal electrophoretic components of casein; however, the recent studies of WAKE AND BALDWIN<sup>1</sup> using starch electrophoresis containing urea have shown that most of these preparations are probably heterogeneous. This finding has made it essential that new methods for separating the components of casein be reinvestigated, using the high resolving method of starch-gel-urea electrophoresis as a method of determining homogeneity. The present paper describes a method of preparing  $\beta$ - and  $\gamma$ -casein, essentially homogeneous by starch-gel electrophoresis in urea, using ion-exchange cellulose chromatography of casein fractions obtained by the acid extraction of casein. Fractionations of casein on anion-exchange cellulose have been reported previously by SCHÖBER AND HEIMBURGER<sup>2</sup> and ZITTLE<sup>3</sup>. Also, PAYENS<sup>4</sup> has found zone electrophoresis on cellulose columns with urea buffer mixtures to give satisfactory separation of casein fractions.

### EXPERIMENTAL

#### *Zone electrophoresis*

Starch-gel electrophoresis was run at pH 8.6 (and also at acid conditions) in 7.0 M urea by the method of WAKE AND BALDWIN<sup>1</sup>. For acid conditions, a solution 0.05 M in formic acid and 0.01 M in sodium hydroxide, pH 3.1, was used in the electrode vessels; and the starch gel was prepared with this buffer made to 5.0 M urea, resulting in a pH 3.8-3.9 as measured on a sample diluted with water (approx. 1-5). The mixture should be heated carefully to minimize urea decomposition. The usual procedure was to heat the suspension rapidly over an open flame with mechanical stirring until the temperature reached 80°.

\* Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

### Preparation of casein

Washed, acid precipitated casein, prepared from 68 l of unpasteurized skim milk, was made to 16 l with water, adjusted to pH 4.0 with 1 *N* acetic acid, as previously described<sup>5</sup> but stirred for 2 h in a cold room (2° instead of 25°). The suspension was then filtered overnight at 2° on large Buchner funnels. The filtrate (9 l) was made to pH 6.0 with dilute sodium hydroxide resulting in a precipitate that was removed by centrifugation at 2° and, when dried, amounted to 18 g of protein. This fraction contained proteolytic activity. The supernatant, on warming to 26°, gave a precipitate that was rich in  $\beta$ - and  $\gamma$ -casein. It was removed by centrifugation at room temperature and on lyophilization weighed 11.0 g. This fraction was used to prepare  $\beta$ - and  $\gamma$ -casein by chromatography on DEAE-cellulose.

### Column chromatography

DEAE-cellulose, type 20, with a capacity of 0.73 mequiv/g was obtained from Brown Company, Berlin, New Hampshire\*. A column 2 × 30 cm was prepared as described by SOBER *et al.*<sup>6</sup> and stepwise chromatography was carried out at 2° using a fraction collector. The column was equilibrated with 0.005 *M*, pH 8.3, sodium phosphate and charged with a concentrated protein solution (about 8%) in phosphate buffer, adjusted to pH 8.3, then dialyzed against 0.005 *M* sodium phosphate 16 h at 2°. Elution in steps of increasing salt concentration and decreasing pH at a flow rate of 24 ml/h was used. Fractions of about 12 ml were collected and examined in a spectrophotometer at 280  $\mu$ . A total of 3 runs were made using about 2 g of protein/run. Protein recovery based on absorbancy or weight amounted to 85–90%. In Fig. 1 an elution diagram for the fraction rich in  $\gamma$ - and  $\beta$ -casein (2.15 g) is shown. The effluent was divided to yield fractions A, B, C, and D as shown by the broken vertical lines and on dialysis, followed by lyophilization, amounted to 0.5, 0.35, 0.7,

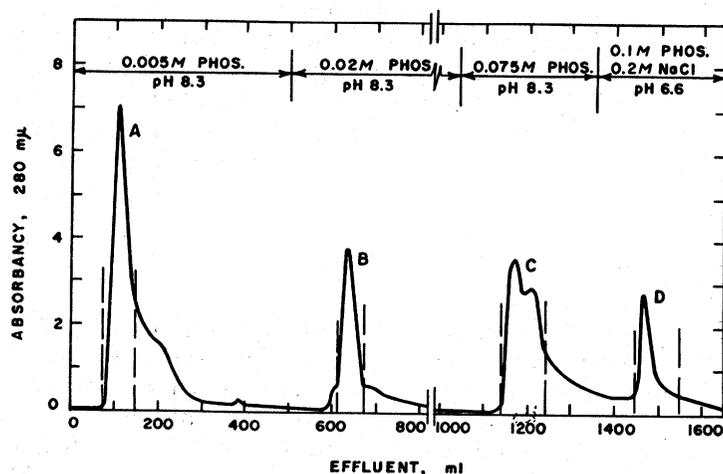


Fig. 1. Stepwise elution diagram of fraction rich in  $\gamma$ - and  $\beta$ -casein; 2.15 g protein in 30 ml applied to a 2 × 30 cm column, effluent collected in about 12 ml fractions.

\* It is not implied the U.S.D.A. recommends the above company or its product to the exclusion of others in the same business.

and 0.2 g of protein, respectively. Starch-gel electrophoresis at pH 8.6, 7.0 *M* urea for the 4 fractions is shown in Fig. 2.

Fraction A, with the slowest mobility, is temperature sensitive: at 2°, pH 8.0, solutions of this fraction remained clear but on warming to 25°, the protein precipitated. Because of this property, aliquots of the effluent solutions of Fraction A were acidified before they were read at 280 *mμ*. A report on this unusual component of casein will be made at a later date. In Fraction B,  $\gamma$ -casein predominates with minor bands preceding and following the major zone. Fraction C is rich in  $\beta$ -casein, C' and C'' (Fig. 2) represent the first and second peaks of C in Fig. 1. Although there may be some chromatographic heterogeneity, differences in zone electrophoresis were small, and the two peaks were combined for rechromatography. In one experiment before elution of Fraction C, the strength of the buffer was changed to 0.05 *M* instead of 0.075 *M* phosphate. However, no improvement in the sharpness of peaks nor homogeneity of products was obtained. The last chromatographic fraction, D, shows a major area with a mobility similar to  $\beta$ -casein together with several minor protein bands. If the buffer change was not made as indicated for Fraction D, it would be included in the long trail of Fraction C. This, together with the electrophoretic data, would suggest that some of the  $\beta$ -casein is tightly complexed with other minor proteins.

A  $\gamma$ -casein fraction corresponding to B, Fig. 1 (0.64 g), was next rechromatographed on DEAE-cellulose with 0.02 *M* sodium phosphate, pH 8.3, as shown in Fig. 3. The major portion enclosed by dashed vertical lines amounted to 0.46 g and was used to determine the physical chemical properties of  $\gamma$ -casein shown below. Paper electrophoresis (Durrum) in veronal buffer of the 3 fractions separated by the dashed lines showed the first fraction to be significantly different. There was a zone corre-



Fig. 2. Zone electrophoresis on starch gel, pH 8.6, 7.0 *M* urea: a, temperature sensitive fraction; b,  $\gamma$ -casein; c,  $\beta$ -casein; d, last fraction (Fig. 1D).

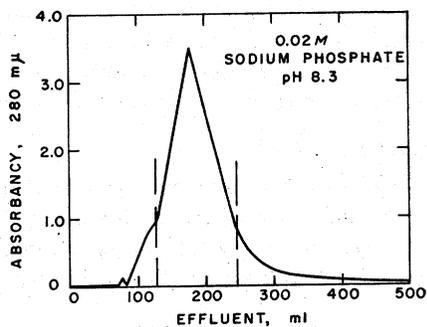


Fig. 3. Effluent diagram on rechromatography of  $\gamma$ -casein (Fig. 1B): 0.64 g protein in 8 ml applied to a 2 × 32 cm column, effluent collected in 8-ml fractions.

sponding to the temperature-sensitive fraction besides the  $\gamma$ -casein band. It was also noted that solutions from this fraction were somewhat temperature sensitive. The two latter fractions gave essentially the same patterns for  $\gamma$ -casein on paper electrophoresis. Total protein recovery was about 90% on a weight basis.

In order to remove minor impurities associated with the  $\beta$ -casein recovered from DEAE-cellulose, Fraction C, Fig. 1, was rechromatographed on carboxymethyl-cellulose; Type 20, CM-cellulose with a capacity of 0.47 mequiv/g was also obtained from Brown Company. A  $2 \times 28$  cm column was used, with a starting buffer of 0.005 *M* sodium phosphate pH 6.2, followed by two stepwise buffer changes. A charge of 0.41 g protein in 13 ml was applied to the column, and fractions were collected and analyzed as already described. Protein recovery was 90% by weight; 82% was eluted with the front, 3% with a 0.05 *M* sodium phosphate buffer, pH 8.3, and 15% with 0.1 *M* sodium phosphate, pH 9.0. The  $\beta$ -casein, eluted with the front was used for physical chemical studies. Paper electrophoresis showed it to be homogeneous while later fractions from the column showed small impurities besides the  $\beta$ -casein. Yields of about 1.5 and 3.0 g of purified  $\gamma$ - and  $\beta$ -casein, respectively, can be expected from the 11 g of starting material.

#### *Successive extractions of casein*

The wet casein remaining on the Buchner funnels following the first extraction, as described above, was investigated further as a source of additional  $\beta$ - and  $\gamma$ -caseins. In one experiment, a second extraction was made by dispersing the casein in 76 l of water at 2°, pH 4.0, allowing the mixture to stand overnight, and filtering as before in the cold room. The filtrate was then saturated with ammonium sulfate, and the precipitated protein, after dialysis followed by lyophilization, amounted to 15.1 g. Chromatography of this material on DEAE-cellulose gave a pattern similar to that shown in Fig. 1. However, the yield of the  $\gamma$ -fraction was reduced considerably. Zone electrophoresis of the large  $\beta$ -fraction, eluted with 0.075 *M* phosphate buffer, is shown in Fig. 5, No. 6. Yields of the  $\beta$ - and  $\gamma$ -casein fractions based on the 15.1 g were about 5.0 and 1.3 g, respectively.

In a second experiment, 600 g of the wet casein (170 g dry) was suspended in 3 l of water, adjusted to pH 4.0 with acetic acid, stirred 24 h at 2°, and then filtered at 2°. Four successive extractions were carried out in this manner, and the protein extracted was precipitated with ammonium sulfate. The total yield was 6.5 g. For comparison with the above experiments, this would be equivalent to 60 g extracted, based on 68 l of milk. Paper electrophoretic patterns of this material do not differ significantly from those of the 15.1 g in the last preparation.

#### *Analytical methods*

Moisture was determined by heating at 70° in a vacuum oven for 4 h; nitrogen by a modification of the A.O.A.C.-Kjeldahl method<sup>7</sup>; phosphorus by the method of SUMNER<sup>8</sup>; and ash after adding magnesium acetate and correcting for magnesium oxide, then subtracting the  $P_2O_5$  calculated from the per cent phosphorus.

#### DISCUSSION

The composition and properties of these preparations of  $\beta$ - and  $\gamma$ -casein are shown in Table I. The nitrogen values for  $\beta$ - and  $\gamma$ -casein are in agreement with those reported

TABLE I  
COMPOSITION AND PROPERTIES OF  $\beta$ - AND  $\gamma$ -CASEIN

	$\beta$ -Casein	$\gamma$ -Casein
N %	15.35	15.81
P %	0.48	0.14
Excess ash* (%)	0.43	0.51
Mobility**, u, pH 8.40	-2.94	-1.96
Mobility, u***, pH 2.35	Insoluble	+2.81
Sedimentation§, pH 7.0, 1% solution	1.17 S (0.8°)	1.15 S (1.3°)
Sedimentation, pH 2.35, NaCl-HCl, 0.1 $\mu$ , 1% solution	Insoluble	6.68, 7.82 (25.2°)

\* Attributed to inorganic contamination.

\*\* Calculated from descending boundary, veronal buffer, 0.05 M NaCl, 0.1  $\mu$ , 1% solution.

\*\*\* Calculated from descending boundary NaCl-HCl, 0.1  $\mu$ , 1% solution.

§ Sodium phosphate buffer, 0.05 M NaCl, 0.1  $\mu$ .

by HIPP *et al.*<sup>9,10</sup>; however, phosphorus values reported here are about 20% lower for  $\beta$ -casein and 30% higher for  $\gamma$ -casein.

Mobilities in veronal buffer are in agreement with published values<sup>9</sup>, and both caseins are electrophoretically homogeneous by the Tiselius method. However, the descending pattern for  $\beta$ -casein from the column is more symmetrical than those shown by HIPP *et al.*<sup>10</sup> and WARNER<sup>11</sup>. Electrophoresis was not carried out on  $\beta$ -casein at acid pH values because of its insolubility. Although  $\gamma$ -casein moves as one component at pH 2.3, NaCl-HCl, 0.1  $\mu$ , with a mobility of 2.81, the peak is unsymmetrical. MURTHY AND WHITNEY<sup>12</sup> report a mobility of 3.23 for  $\gamma$ -casein at the same pH in 0.1  $\mu$ , glycine-HCl buffer.

A single sedimentating boundary was observed for  $\beta$ -casein at pH 7.0, 0.8°, with a sedimentation coefficient of 1.17 S. This compares with a value of 1.27 S calculated from data of SULLIVAN *et al.*<sup>13</sup> and 1.30 S at 6.5° reported by McMEEKIN AND PETERSON<sup>14</sup>. PAYENS' preparation of  $\beta$ -casein by zone electrophoresis on a cellulose column had an  $s_{20}$  value of 1.65 S; however, free electrophoresis showed some minor contamination and the reported mobility of -3.87 in veronal buffer, 0.1  $\mu$ , pH 7.5, is also considerably higher than the literature values. The sedimentation of  $\gamma$ -casein at pH 7.0 gave a single boundary with an  $s_{20}$  of 1.15 S. This is somewhat lower than the 1.41 S determined at pH 8.6 and reported by MURTHY AND WHITNEY. Different values of 12.6 S and 1.4 S for major and minor components of  $\gamma$ -casein in phosphate buffer, pH 7.2, 0.2  $\mu$ , 4°, have been reported by PANTLITSCHKO AND GRÜNDIG<sup>15</sup>. At pH 2.3, 25°,  $\gamma$ -casein shows two peaks with about 80% of the area of the pattern under the 7.82 S peak. MURTHY AND WHITNEY find that at the same pH and 5°  $\gamma$ -casein possesses at least 3 components sedimentating close to one another, the major one 67%, with an  $s_{20}$  value of 9.72.

Further comparison of the various preparations was made by starch-gel electrophoresis. In Fig. 4 are shown the patterns of  $\beta$ -casein prepared by various methods run at pH 8.6, 7.0 M urea (b) and at pH 3.9, 5.0 M urea (a).  $\beta$ -Caseins prepared earlier by WARNER (Run No. 1) and HIPP (Run No. 2) show heavy trailing at both acid and alkaline pH values while with the urea  $\beta$ -casein preparation (No. 3) trailing is greatly reduced. Both the alcohol and urea  $\beta$ -caseins had also been further purified by the Warner method. The urea  $\beta$ -casein was then fractionated on DEAE-cellulose, as

described above, and the major fraction eluted with 0.075 *M* phosphate, pH 8.3, is shown in No. 4 while No. 5 represents the combined material eluted with buffers of higher salt and lower pH. This latter fraction amounted to about one-third of the material put on the column and shows a considerable amount of trailing, especially at pH 8.6, and a faster-moving band at acid pH, which is not seen in the original urea preparation (No. 3). Phosphorus determined on the main fraction eluted with 0.075 *M* phosphate buffer was about 7% lower than that of the original urea  $\beta$ -casein No. 6 shows the pattern of  $\gamma$ -casein prepared by HIPP *et al.*<sup>9</sup>

Starch-gel-urea runs for two samples of  $\beta$ -casein prepared by chromatography on both DEAE- and CM-cellulose, as described in this paper, are shown in Fig. 5, Nos. 4 and 5. No. 6 shows the  $\beta$ -casein prepared by the modified method of extraction involving a larger volume of water. It had not been rechromatographed and shows a minor faster-moving band at acid pH. No. 1 is  $\gamma$ -casein prepared earlier by HIPP<sup>9</sup> and Nos. 2 and 3 show two preparations of  $\gamma$ -casein by the present method.

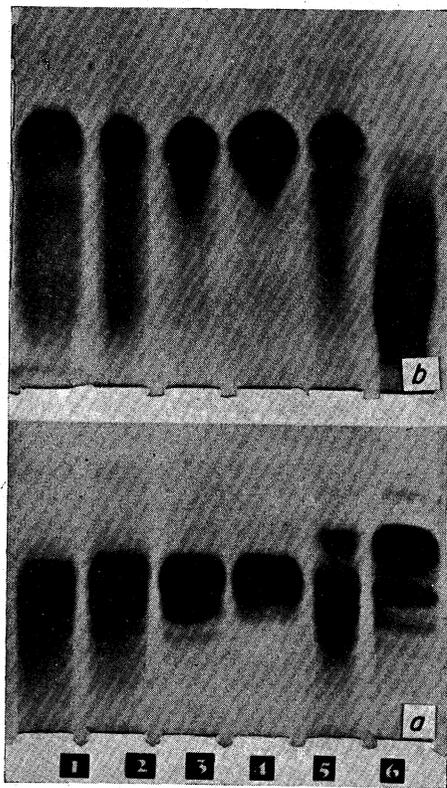


Fig. 4. Starch-gel-urea patterns (a, pH 3.9) b, pH 8.6) of caseins prepared by previous methods: (1)  $\beta$ -casein (WARNER) cold room method. (2)  $\beta$ -casein (HIPP) alcohol and cold room method. (3)  $\beta$ -casein (HIPP) urea and cold room method. (4)  $\beta$ -casein (3) after DEAE-cellulose. (5)  $\beta$ -casein (3) after DEAE-cellulose. (6)  $\gamma$ -casein (HIPP).

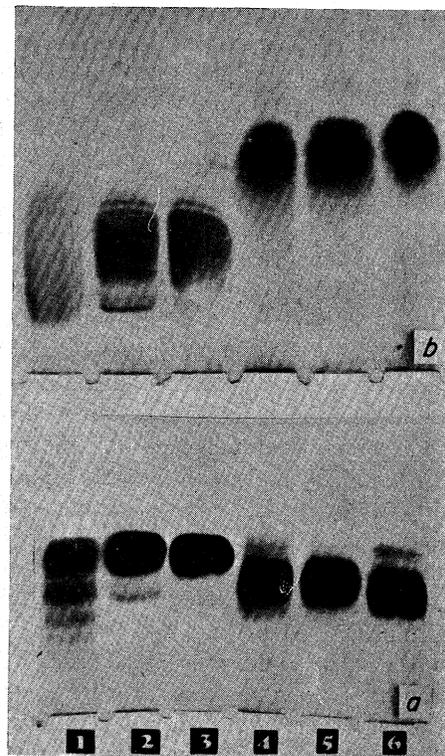


Fig. 5. Starch-gel-urea patterns (a, pH 3.9) b, pH 8.6) of  $\beta$ - and  $\gamma$ -casein prepared by DEAE-cellulose together with an early preparation of  $\gamma$ -casein. (1) Early preparation  $\gamma$ -casein. (2)  $\gamma$ -casein (DEAE). (3)  $\gamma$ -casein (DEAE). (4)  $\beta$ -casein (DEAE). (5)  $\beta$ -casein (DEAE). (6)  $\beta$ -casein (DEAE).

Both  $\beta$ - and  $\gamma$ -casein are homogeneous at pH 3.9 (a), as shown by Nos. 3 and 5 respectively, while at pH 8.6 (b) some heterogeneity is evident. In contrast to the present preparations, early  $\beta$ - and  $\gamma$ -casein samples show heavy trailing and multiple slower-moving bands. The fact that the casein preparations made by earlier methods have been stored in a powder form for years may have been a source of their heterogeneity; however, unpublished experiments indicate that the previously described methods do not produce homogeneous casein preparations as determined by starch-gel electrophoresis in urea. Since experimental evidence supports the hypothesis that molecular size plays an important role in determining mobilities in starch gel<sup>16</sup>, the heavy adsorption of early preparations may represent larger aggregates of the same protein.

It is interesting to note that preliminary amino acid analyses of the new preparations of  $\beta$ - and  $\gamma$ -casein show only minor differences from published values except in the case of arginine which is significantly lower for the new preparations. Additional analyses are in progress and will be reported elsewhere.

The lower phosphorus content (20%) for the  $\beta$ -casein prepared by this new method is significant. This could be due to the presence of a  $\beta$ -casein with a lower phosphorus content than the principal  $\beta$ -casein component or to the loss of phosphorus during preparation by the action of enzymes present in the acid extract. A second acid extraction of the casein using a larger volume followed by chromatography on DEAE-cellulose gave a  $\beta$ -casein preparation containing 13% less phosphorus than the usual value. When  $\beta$ -casein, prepared by the urea method, was fractionated on DEAE-cellulose, the phosphorus content of the main component was 7% less than the original preparation. While these results indicate the possibility of the phosphorus content of  $\beta$ -casein being somewhat lower than the published value, they do not account for the low value of 0.48% by the new method. Further experimentation is required to clear up this point.

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