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CHEMICAL AND CHROMATOGRAPHIC ISOLATION OF *K*-CASEIN*,**

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SUMMARY

Improved chemical and chromatographic methods for the isolation of electrophoretically pure *K*-casein have been developed. The chemical method is simple, mild, reproducible, and can be completed in about one day. Contact of the protein with reactive chemicals is minimized. The use of 6.6 *M* urea in the first step apparently does not adversely affect the *K*-casein. The remaining treatments, addition of Ca^{2+}, pH adjustment, dialysis, and centrifugation are mild. The method gives a good yield of *K*-casein (1.5 g/15 g of pI casein). A very pure *K*-casein was obtained by DEAE-cellulose chromatography of the chemically isolated protein. The polyacrylamide and starch gel urea electrophoretic gel patterns of chemically prepared *K*-casein show that the protein is electrophoretically pure and the gel zone patterns of *K*-casein reduced with 2-mercaptoethanol show the characteristic bands as reported by others. The amino acid composition, and content of sialic acid and phosphorus are comparable with the reported values. A single symmetrical sedimenting peak of 13.5 S was obtained on ultracentrifugation in 0.076 *M* Tris–0.005 *M* citrate buffer, pH 8.6.

INTRODUCTION

In protein research, the important objective is to obtain a preparation in pure form. The isolation procedure should be simple, involve mild treatment of the protein, and give a good yield of the final preparation. DEAE-cellulose column chromatography and Sephadex gel filtration have been used as the major means of purifying the caseins[1–3]. One of the most common chemical fractionation procedures in use for isolation of the *K*-casein is the urea–sulfuric acid method[4], which involves the use of 7 *N* H_2SO_4. The *K*-casein obtained by this method contains major impurities of

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α_{s1}- and β-caseins. A further purification is usually made by ethanol precipitation[5].

This research reports a simple, reproducible, two-step chemical procedure for isolation of K-casein, chromatography on DEAE-cellulose and comparisons with K-caseins isolated by other chemical and chromatographic methods. Characterization was by polyacrylamide (PGUE), and starch gel urea electrophoresis (SGUE), chemical and amino acid analyses, sedimentation velocity, and gel-zone electrophoresis after reduction with 2-mercaptoethanol (2ME).

EXPERIMENTAL

The procedures for the preparation of the K-casein are outlined as follows and in the accompanying flow diagram. The essentials of the isolation of K-casein include the urea fractionation of isoelectric casein into the α_{s1}-K-casein complex with β- and γ-casein remaining in the supernatant. The α_{s1}-K-casein complex is then fractionated with 0.25 M $CaCl_2$ at pH 7.0, followed by DEAE-cellulose column chromatography for further purification.

FLOW DIAGRAM

CHEMICAL FRACTIONATION OF K-CASEIN

Isoelectric casein (15 g) from pooled milk, twice precipitated at pH 4.6 and washed.

1 — Disperse in 400 ml H_2O. Dissolve in 6.6 M urea, dilute to 4.6 M urea. Allow to stand 8 h at 2°, pH 4.6 Centrifuge at 3000 r.p.m. for 30 min.

- Supernatant (β- and γ-casein). Use for preparation of β- and γ-casein.
- Precipitate (α_{s1}-K-casein gel).

2 — Repeat step 1.

- Supernatant. Discard.
- Precipitate (α_{s1}-K-casein gel).

3 — Disperse in 250 ml H_2O. Add 0.1 N NaOH dropwise with stirring to pH 8.5. Adjust to pH 7.0 with 0.1 N HCl. Make 0.25 M in $CaCl_2$ with 4 M $CaCl_2$. Maintain at pH 7.0 with 0.1 N NaOH. Centrifuge at 3000 r.p.m. for 30 min.

- Precipitate Ca-α_{s1}-, and trace of β-casein. Use for α_{s1}-casein preparation.
- Supernatant (enriched-K-casein).

Dialyze, lyophilize and repeat step 3.

4

- Precipitate. Discard.
- Supernatant (enriched K-casein).

5 — Centrifuge at 30,000 r.p.m. for 30 min.

- Precipitate (Ca-α_{s1}-casein). Discard.
- Supernatant (K-casein).

6 — Dialyze *vs.* distilled H_2O at 2° using 4 changes at 4 to 8 h each.

Chemically prepared K-casein.
Further purified by DEAE-cellulose chromatography.

Chemical method

Fifteen grams of isoelectric casein were suspended in 400 ml of distilled water and dissolved in urea solution (6.6 M). The solution was diluted to 4.6 M in urea and

held until the precipitate settled (approximately 8 h). The supernatant, containing the β- and γ-caseins, was decanted and discarded. The precipitate, containing the α_{s1}-*K*-casein complex, was washed with distilled water and again dissolved in urea solution (6.6 *M*). The solution was diluted to 4.6 *M* in urea. The supernatant was decanted and discarded. The gelatinous α_{s1}-*K*-casein complex was dispersed in 250 ml of distilled water and was dissolved by the dropwise addition of 0.1 *N* NaOH with constant stirring to pH 8.5, which was not exceeded. The solution was readjusted to pH 7.0 with 0.1 *N* HCl, and sufficient 4 *M* $CaCl_2$ was slowly added to make the resulting solution 0.25 *M* in $CaCl_2$. The pH was maintained at 7.0 by the addition of 0.1 *N* NaOH. The suspension was centrifuged at 3000 r.p.m. for 30 min. The supernatant (*K*-casein) was saved and the precipitate, Ca-α_{s1}- and β-caseinates, was discarded. The supernatant was thoroughly dialyzed until free of Ca^{2+}, lyophilized, and then the $CaCl_2$ treatment was repeated as above. The suspension was centrifuged at 30,000 r.p.m. for 30 min. The supernatant, containing the *K*-casein, was dialyzed against distilled water until free of Ca^{2+}, then lyophilized. All of the operations were carried out at 2–4°. The yield was at least 1.5 g of electrophoretically pure *K*-casein.

The chemically prepared *K*-casein was purified by both DEAE-cellulose column chromatography and 75% ethanol 1 *M* in NH_4Ac methods to determine if an improvement in the purity of the isolated *K*-casein could be obtained.

Chromatography on DEAE-cellulose

DEAE-cellulose with 0.70 mequiv./g exchange capacity, obtained from Bio-Rad Laboratories, was used. The anion exchanger was regenerated and thoroughly washed. A 25 × 4.5 cm column was used, and equilibrated with 2 l of 0.03 *M* glycine–NaOH buffer (pH 8.0) containing 2 *M* urea, at a flow rate of 250 ml/h. A soft gradient stepwise elution was made with successively higher concentrations of NaCl (0.05–0.25 *M*) in the glycine–NaOH buffer. The effluent was continuously monitored and recorded by a Vanguard automatic UV analyzer, Model 1056. The effluent fractions were dialyzed against distilled water for 24 h with changes each 4 h, then lyophilized. In addition, the methods of ZITTLE AND CUSTER[4] and MCKENZIE AND WAKE[5] were used for the isolation of *K*-casein preparations which were then compared with the chemically and chromatographically prepared *K*-casein described in this paper. Also, the ethanol–NH_4Ac method of MCKENZIE AND WAKE[5] was used for further purification of the chemically prepared *K*-casein.

PGUE and SGUE characterization

The prepared *K*-caseins were characterized by PGUE and SGUE. The details of the electrophoresis, gel concentration, sample loading, and buffer conditions, etc., have been described by GEHRKE *et al.*[6]. The chemically and chromatographically prepared *K*-caseins were also reduced with 2ME (0.2 v/v%) in Tris (hydroxymethyl)-aminomethane (THAM) Na_2EDTA–boric acid buffer pH 9.2 for PGUE. The same concentration of 2ME in Tris–citrate buffer pH 8.6 was used for SGUE. A comparative study of the *K*-caseins prepared by the different methods was made by PGUE.

Amino acid analysis

The amino acid composition of the *K*-casein preparation was determined with a Technicon automatic amino acid analyzer using the procedure described by PIEZ

AND MORRIS[7]. Thirty milligrams of duplicate samples, dried over P_2O_5 for several hours, were hydrolyzed for 24 h with 6 *N* HCl at 110° in a sealed tube under nitrogen. The HCl was removed with rotary evaporation at room temperature or by lyophilization. The separations were made on a 0.6 × 133 cm column of 22 micron spherical polynuclear sulfonic acid resin beads (Chromobeads A) at 60° using citrate buffers of pH 2.875, 3.80 and 5.00 for the gradient elution. The data are given in Table I.

TABLE I

WEIGHT PER CENT OF AMINO ACIDS IN *K*-CASEIN

Each value is an average of 2 independent determinations. Chemical preparations.

Amino acid	*Per cent*	*Amino acid*	*Per cent*
Aspartic acid	7.94	Methionine	1.92
Threonine	6.64	Isoleucine	5.98
Serine	5.63	Leucine	6.04
Glutamic acid	19.03	Tyrosine	7.13
Proline	7.88	Phenylalanine	3.52
Glycine	1.17	Lysine	7.18
Alanine	4.86	Histidine	2.18
Valine	5.36	Arginine	3.65
Half-cystine	0.75		
		Total	96.86

Chemical analysis

The sialic acid content of the *K*-casein was determined by WARREN's thiobarbituric acid method[8]. Thirty milligrams were hydrolyzed at 80° for 1 h in 10 ml 0.1 *N* H_2SO_4 to release the sialic acid. Sialic acid (concentrated assay, 16.5% sialic acid) was used as the standard. The standards and unknown were carried through the same procedure.

The phosphorus content was determined by the phosphomolybdovanadate method. The standards were prepared to contain 0.5, 1.0, 1.5, and 2.0 mg of P_2O_5/100 ml from KH_2PO_4. The standards and samples were read on a Beckman Model DU spectrophotometer at 400 nm against a distilled water blank.

Sedimentation analysis

The chemically prepared *K*-casein was studied by means of ultracentrifugation. The sedimentation velocity experiments were made at 20° with a rotor speed of 59,780 r.p.m. A valve type 4°/12 mm synthetic boundary cell was used.

RESULTS AND DISCUSSION

The PGUE patterns of the chemically prepared *K*-casein and *K*-caseins obtained by four other different methods are presented in Fig. 1. The sample loading for the chemically prepared *K*-casein was 0.7 mg and for the other preparations 0.25 mg each. It is noteworthy that the gel zone patterns for the chemically prepared *K*-casein at about 3 times the loading showed absence of contamination, as was observed for the chromatographically[9] or ethanol–NH_4Ac purified samples (Fig. 1). Further, the chemically prepared *K*-casein gave nearly the same gel zone pattern as for the chro-

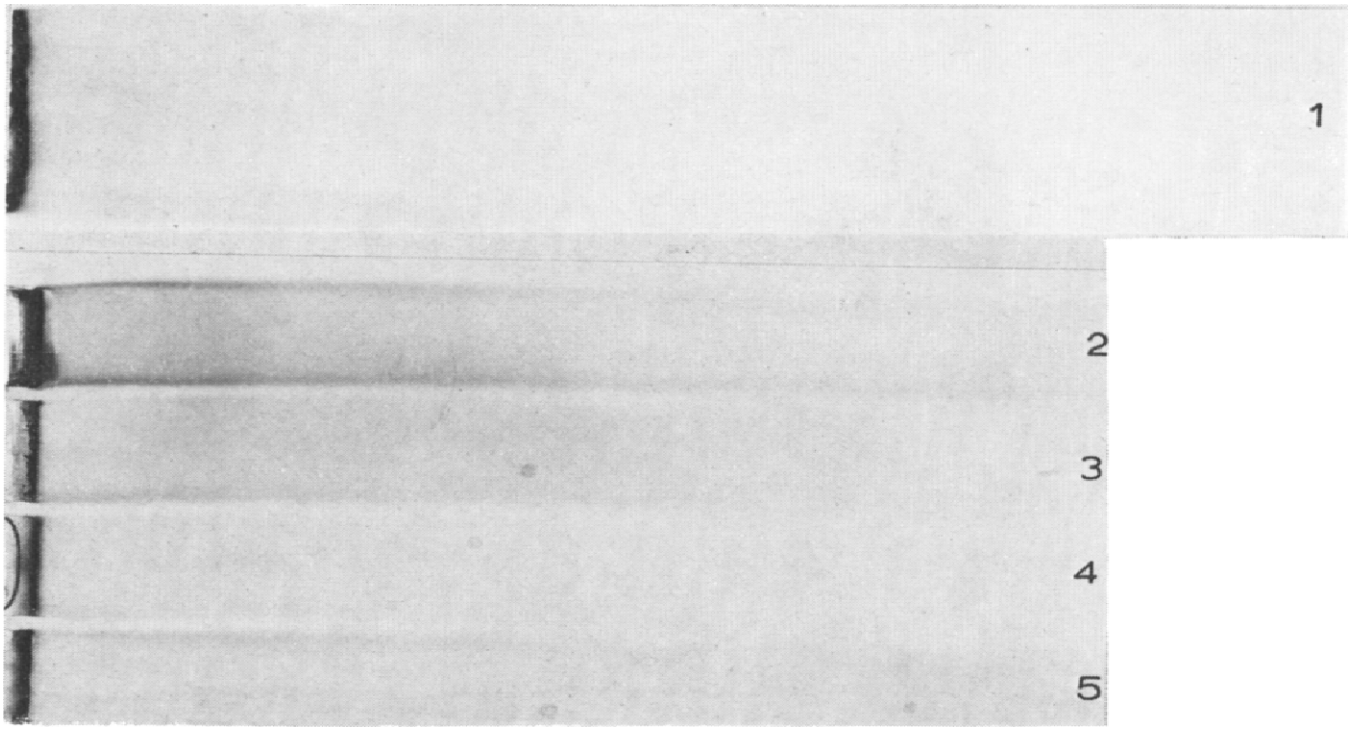

Fig. 1. PGUE patterns of *K*-casein. *K*-casein prepared by different methods. 1 = chemically prepared; 2 = chemically prepared, purified by ethanol–ammonium acetate precipitation; 3 = purified by DEAE-cellulose column chromatography; 4 = McKenzie and Wake[5]; 5 = Zittle and Custer method, with ethanol–NH_4Ac purification[4]. PGUE conditions: 7% cyanogum, 4.5 *M* urea, pH 9.2. Slot 1: 0.7 mg sample. Nos. 2–5, 0.25 mg. Run vertically for 5 h, 200 V, at 18°. Buffer was 0.083 *M* in THAM, 0.003 *M* in Na_2EDTA, and 0.012 *M* in H_3BO_3.

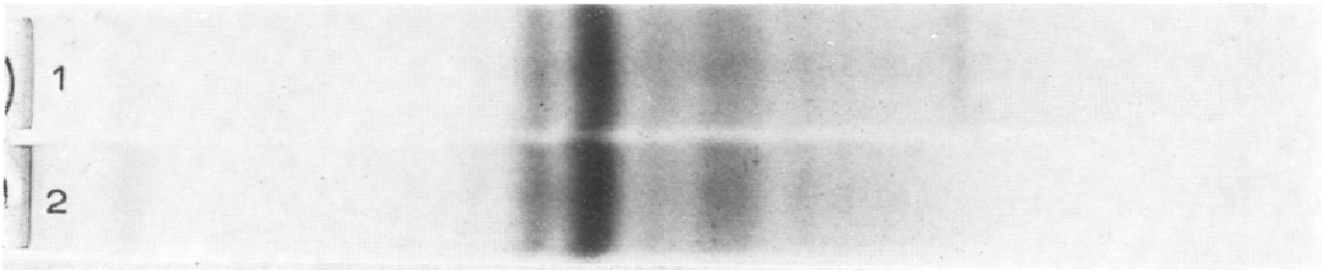

Fig. 2. PGUE patterns of *K*-casein. *K*-casein reduced with 2ME. 1 = Chemically prepared; 2 = purified by DEAE-cellulose column chromatography. PGUE conditions: 7% cyanogum, 4.5 *M* urea, pH 9.2, 0.2% 2ME, 0.7 mg sample. Run vertically for 5 h, 200 V, at 18°. Buffer was 0.083 *M* in THAM, 0.003 *M* in Na_2EDTA, and 0.012 *M* in H_3BO_3.

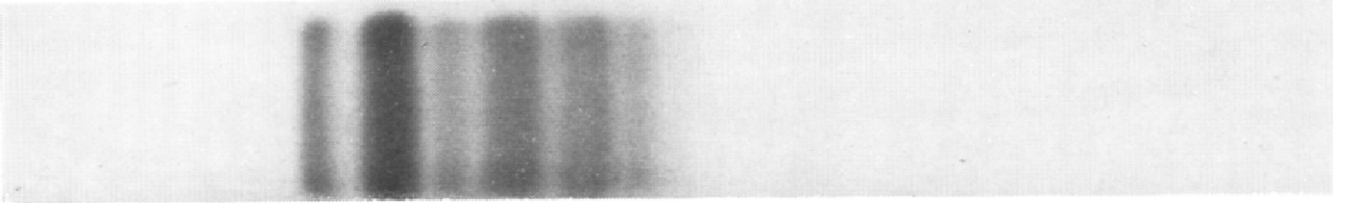

Fig. 3. SGUE pattern of *K*-casein. *K*-casein reduced with 2ME. SGUE conditions: 16% starch, 6 *M* urea, pH 8.6, 0.2% 2ME, and 0.7 mg sample. Gel buffer was 0.076 *M* Tris–0.005 *M* citrate and the bridge buffer was 0.3 *M* Na-borate. Run vertically for 18 h, 200 V, at 5°.

TABLE II

CHEMICAL ANALYSES OF *K*-CASEIN

Each value is an average of 4 independent analyses. Chemical preparations.

	NANA	*Phosphorus*
Per Cent[a]	1.89 ± 0.02	0.32 ± 0.01

[a] Based on sample vacuum dried over P_2O_5.

matographically purified *K*-casein when reduced with 2ME (Fig. 2). The SGUE pattern of the chemically prepared *K*-casein reduced with 2ME (Fig. 3) shows the characteristic bands for *K*-casein as reported in the literature. All of the preparations were considered electrophoretically pure.

The amino acid composition (Table I), and the sialic acid and phosphorus content (Table II) of the chemically prepared *K*-casein are in agreement with the reported values for *K*-casein.

The analytical ultracentrifuge pattern of chemically prepared *K*-casein is shown in Fig. 4. A single peak was observed. The sedimentation coefficient in 0.076 *M* Tris–0.005 *M* citrate buffer at pH 8.6 was found to be 13.5 S.

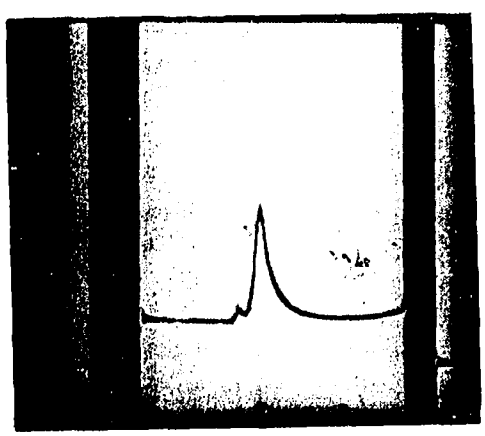

Fig. 4. Ultracentrifuge pattern of *K*-casein. Sedimentation of *K*-casein at 59,780 r.p.m., using a Valve Type synthetic boundary cell with 4°/12 mm sector, temperature 20°, 0.076 *M* Tris–0.005 *M* citrate buffer, pH 8.6, 0.7% chemically prepared *K*-casein, bar angle 60°, 1500 sec.

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