Purification of $\alpha_{s1}$-Casein by Fast Protein Liquid Chromatography

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ABSTRACT
An enriched $\alpha_{s1}$-casein was prepared by batch fractionation of whole casein on DEAE-cellulose using $\text{CaCl}_2$ as eluent. The $\alpha_{s1}$-casein fraction was then submitted to hydrophobic interaction chromatography on a semipreparative fast protein liquid chromatography system. Electrophoretically pure $\alpha_{s1}$-casein was obtained by eluting with .05 M sodium phosphate at pH 6 and containing 3.75 M urea.

INTRODUCTION
Whole casein (CN) is commonly fractionated using anion-exchange chromatography on DEAE cellulose columns (6, 10, 12, 13, 14, 15, 17). During recent years HPLC gave rise to new developments with modern anion-exchangers (1, 2, 4, 6, 7). Other HPLC techniques, e.g., reverse-phase chromatography (2), have been developed on an analytical scale to fractionate CN. More recently, hydrophobic interaction chromatography has also been used (3). The objective was to achieve large yields of bovine pure $\alpha_{s1}$-CN. In this way, whole CN was submitted to batch fractionation on an ion exchanger to separate prepurified $\alpha_{s1}$-CN. Hydrophobic interaction chromatography adapted to semi-preparative fast protein liquid chromatography (FPLC) was then used to obtain pure $\alpha_{s1}$-CN.

MATERIALS AND METHODS

Reagents
Acrylamide and bisacrylamide were from BDH Poole, England. The DEAE-cellulose DE 52 was from Whatman LTD, Springfield, England. Analytical grade urea and 2-mercaptoethanol were from Merck, Darmstadt, FRG.

Whole Casein
Skim milk was separated from raw bulk milk by centrifugation (4000 x g, 30 min, 30°C) and whole CN was precipitated by adjusting the pH to 4.6 with 1 N HCl. After centrifugation (4000 x g, 30 min) the precipitate was washed with water and dissolved with 1 N NaOH at pH 7.5. The washing at pH 4.6 was repeated once, and whole CN was dissolved at pH 7.5 with 1 N NaOH, then lyophilized.

Batch Fractionation of Whole Casein
The batch fractionation method described by Wei and Whitney (16) was modified. The imidazole-NaCl, pH 7, buffer system was substituted by acetate-$\text{CaCl}_2$ at pH 6.6 and EDTA was added in the equilibration buffer of the ion exchanger and in the CN solution. The procedure is summarized in Figure 1. Whole CN was dispersed in .02 M sodium acetate buffer pH 6.6, containing 3.3 M urea, .035 M EDTA, .01 M 2-mercaptoethanol, then mixed with one volume of the equilibrated DEAE cellulose suspension to give a final ratio of 5 g of casein to 20 g of dry DEAE-cellulose. After shaking the mixture was filtered through Whatman No. 41 filter paper. The DEAE-cellulose filter cake was then dispersed in 200 ml of the same buffer and the procedure was repeated twice. Subsequent casein fractions were obtained by adding increasing $\text{CaCl}_2$ concentrations up to .05 M in the acetate buffer free from EDTA.

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Whole casein (5 g in 100 ml of buffer-urea-EDTA system)

100 ml DEAE-cellulose (20 g dry weight) in buffer-urea-EDTA system

Regeneration

Filter cake

Filtrates (Fraction 4)

CaCl$_2$ .0375 M

Filter cake

CaCl$_2$ .0275 M

Filter cake

CaCl$_2$ .0125 M

Filtrates (Fraction 1)

Shake 15 min at 20°C and filter
Two additional extractions with buffer-urea system

Filtrates (Fraction 2)

Add 200 ml buffer-urea system without EDTA
Shake 15 min at 20°C and filter
Two additional extractions with buffer-urea system

Filtrates (Fraction 5)

CaCl$_2$ .05 M

Filter cake

Crude casein (Fraction 1)

Two additional extractions with buffer-urea system

Figure 1. Schematic presentation of whole casein batch fractionation on DEAE cellulose.

Figure 2. Urea-PAGE patterns of casein fractions obtained by batch fractionation on DEAE cellulose. W = Whole casein; 1, 2, 3, 4, and 5 = fractions (filtrates) obtained from .0, .0125, .0275, .0375, and .05 M CaCl$_2$ extraction, respectively.

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The filtrates were dialyzed against ultrapure water and lyophilized.

Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography was carried out on a FPLC system (Pharmacia, Uppsala, Sweden) fitted with a semipreparative Spherogel TSK-G Phenyl 5 PW (2.15 x 15 cm) column (Beckman Instruments, Palo, San Alto, CA). Prepurified \( \alpha_\text{S1}-\text{CN} \) prepared by batch fractionation (100 mg) was dispersed in 2 ml of \( .2 \text{M} \) sodium phosphate, pH 6.0 containing 3.75 \( \text{M} \) urea, filtered, then loaded on the Phenyl 5 PW column previously equilibrated in the same buffer. A first washing step was performed with the same buffer at a flow rate of 6 ml/min to elute the unadsorbed proteins in the first two peaks. The \( \alpha_\text{S1}-\text{CN} \) was then eluted with \( .05 \text{M} \) sodium phosphate buffer pH 6.0 containing 3.75 \( \text{M} \) urea. The fractions obtained were dialyzed against ultrapure water and lyophilized.

Polyacrylamide Gel Electrophoresis

Each filtrate and chromatographic fraction was submitted to urea-PAGE (11). In this way, 80 \( \mu \text{l} \) of each fraction containing \( .01 \text{M} \) 2-mercaptoethanol and 5% glycerol (vol/vol) were analyzed.

RESULTS AND DISCUSSION

Batch Fractionation

During ion-exchange chromatography the anionic and cationic components of the eluent are both effective (8, 9). The chloride anion was the counter ion. In the present study phosphoproteins are to be separated. Therefore, calcium cations interact with the phosphorylated groups and contribute to protein separation. Consequently lower concentration of \( \text{CaCl}_2 \) (\( .05 \text{M} \)) was required to elute caseins compared with \( .175 \text{M NaCl} \) used (16). The EDTA added in the protein preparation chelates the calcium naturally bound to casein. We currently use this method because of its high yields in pure \( \beta\)-CN and prepurified \( \alpha_\text{S1}-\text{CN} \). According to the Figure 1, the whole CN was separated in 5 fractions during batch fractionation. Individual CN were eluted in the order commonly observed during classical anion exchange chromatography. As shown on the PAGE patterns (Figure 2) the first fraction contained \( \beta\)-CN X-1P(29-209), X(f106-209), X(f108-209), and \( \kappa\)-CN. The second fraction consisted of a mixture of \( \kappa\)-CN and \( \beta\)-CN. The third fraction, extracted with \( .0275 \text{M CaCl}_2 \), contained highly pure \( \beta\)-CN. The \( \alpha_\text{S2}-\text{CN} \) was separated with residual \( \beta\)-CN in the fourth fraction. The fifth fraction, extracted with \( .05 \text{M CaCl}_2 \), was composed of mainly \( \alpha_\text{S1}-\text{CN} \) with slight contamination from \( \beta\)-CN. From an initial 5 g of whole casein, 1.52 g of prepurified protein were recovered in fraction 5 after lyophilization; 67.5% (wt/wt) of the total \( \alpha_\text{S1}-\text{CN} \) present in 1 L of milk could be recovered. On the average, there are 30 g of whole casein and 13.5 g of \( \alpha_\text{S1}-\text{CN} \) in 1 L of milk (5).

Hydrophobic Interaction Chromatography

An analysis of bovine milk proteins by hydrophobic interaction FPLC was recently published (3). Whole CN was separated using decreasing gradient of .8 to .05 \( \text{M} \) sodium phosphate and a constant 3.75 \( \text{M} \) urea concentration at pH 6.0. The order of elution was...
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\(\beta\text{-CN} < \alpha_{s2}\text{-CN}, \beta\text{-CN X-1P}(f29-209), \beta\text{-CN X}(f106-209), \beta\text{-CN X}(f108-209), < \kappa\text{-CN} < \alpha_{s1}\text{-CN}\). We have adapted this procedure for a semipreparative scale purification of \(\alpha_{s1}\)-CN with an isocratic elution.

Previous assays led us to choose the most satisfactory buffer system consisting of a \(0.2\) M sodium phosphate containing \(3.75\) M urea at pH 6.0. The lyophilized prepurified \(\alpha_{s1}\)-CN obtained from batch fractionation was dissolved in this buffer and loaded on the column. A simple isocratic elution was enough to separate \(\alpha_{s1}\)-CN from the contaminating proteins (\(\beta\), \(\alpha_{s2}\)-caseins, and degradation products), which were eluted in the first two peaks (Figures 3 and 4). However, to accelerate the elution of \(\alpha_{s1}\)-CN and to decrease the elution volume, the change of the buffer molarity from \(0.2\) to \(0.05\) M, just after the elution of the first two peaks, is recommended (Figure 3). Yield of \(\alpha_{s1}\)-CN was 75\% (5 h were required to obtain 375 mg of \(\alpha_{s1}\)-casein after five chromatographies of 100 mg protein each).

CONCLUSION

The combination of anion-exchange batch fractionation with hydrophobic interaction chromatography was efficient for the purification of \(\alpha_{s1}\)-CN from whole CN. The first step of batch fractionation allowed the preparation in a short time of large quantities of prepurified \(\alpha_{s1}\)-CN. The adsorption capacity of \(\alpha_{s1}\)-CN on the ion exchanger and on the hydrophobic ma-

Figure 4. Urea-PAGE patterns of fractions isolated during the purification of \(\alpha_{s1}\)-casein by hydrophobic interaction chromatography. W = Whole casein; a, b, c = fractions eluted with \(0.2\) M sodium phosphate buffer pH 6.0, \(3.75\) M urea; d and e are fractions eluted with \(0.05\) M sodium phosphate buffer pH 6.0, \(3.75\) M urea; s is the fraction loaded on the column.
trix were maximal. Because the elution orders of the other caseins vary, the $\alpha_{52}$- and $\beta$-CN present in the prepurified $\alpha_{51}$-CN were easily eliminated during hydrophobic interaction chromatography. From 1.52 g of prepurified $\alpha_{51}$-CN prepared by batch fractionation of 5 g of whole casein, we obtained 1.14 g of pure $\alpha_{51}$-CN. The second chromatographic step requires only a simple isocratic elution and would be easily extended to a preparative HPLC system.

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