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SEPARATION OF BOVINE CASEINS USING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

All of the casein components could be adsorbed onto Octyl-Sepharose CL-4B from a phosphate buffer solution and separated chromatographically using a gradient of 0–6 *M* urea. β-Casein, despite its higher average hydrophobicity, was eluted first. The large peptides α_{s1}-I and β-I (derived from α_{s1}- and β-casein by chymosin action) were readily separated from their precursor proteins using this technique, and the rare A variant of α_{s1}-casein was well resolved from the commoner B variant.

INTRODUCTION

The caseins of bovine milk constitute nearly 80 % of the total milk proteins and one of their predominant characteristics is their strong association with one another. This association is partly caused by hydrophobic interactions between the proteins[1,2], and it might be expected that the caseins could be separated, one from another, by hydrophobic interaction chromatography[3,4]. Hydrophilic Sepharose gels substituted with hydrophobic groups, such as *n*-octyl groups, have been shown to interact with the surface hydrophobic regions of globular proteins, and these gels have been used to isolate and purify otherwise recalcitrant proteins[5] and for the surface characterization of the surface hydrophobicity of bacteria[6] and proteins[7]. Hydrophobic interaction chromatography was therefore explored as a possible technique for the separation of the bovine caseins from one another.

EXPERIMENTAL

Materials

Phenyl- and Octyl-Sepharose CL-4B were purchased from Pharmacia, Uppsala, Sweden. All other chemicals were reagent grade, and the water was purified with a Milli-Q ion-exchange and filter system (Millipore, Bedford, MA, U.S.A.).

Method

Column chromatography. The Octyl-Sepharose CL-4B beads were packed into a 1.6-cm diameter column to a depth of 25–30 cm in a 0.1 *M* solution of Na_2HPO_4

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adjusted to pH 7.0. The sample (0.1–1.0 g) was applied in 5–10 ml of 0.1 *M* Na_2HPO_4 pH 7.0 buffer. The column was eluted with 200 ml of 0.1 *M* Na_2HPO_4 pH 7.0 buffer at a flow-rate of 160 ml/h maintained with a Pharmacia P-3 peristaltic pump, followed by a linear gradient of 0–6 *M* urea (300 ml each solution) in 0.1 *M* Na_2HPO_4, then 200 ml of 6 *M* urea in 0.1 *M* Na_2HPO_4 and finally 200–400 ml of 0.1 *M* Na_2HPO_4 buffer. The column effluent was fractionated into 20.0-ml fractions using an LKB Ultrorac fraction collector. The absorbance of each fraction was determined at 280 nm, and its casein composition determined by disc gel electrophoresis followed by densitometry using the procedure of Creamer and Berry[8]. Para-κ-casein was determined with the aid of an sodium dodecyl sulfate (SDS) gel system[9].

Chymosin degradation of whole casein. A sample of whole casein[8] was dissolved using 0.1 *M* NaOH to give a 5% solution at pH 6.3. This solution was held at 30°C and mixed with commercial rennet (N.Z. Co-operative Rennet Company, Eltham, New Zealand) to give a rennet concentration of 0.1%. At selected times between 2 min and 16 h, 20-ml samples of the mixture were heated in a boiling-water bath to denature the enzyme. Gel electrophoresis was used to determine the extent of chymosin hydrolysis of the caseins.

Hydrophobicity estimations. The average hydrophobicity per residue was determined using the procedure of Bigelow[10]. The distribution of hydrophobicity along the protein sequence was determined from the sequences of the bovine caseins[11] by a moving-average smoothing technique in which the hydrophobicity of each and every nonapeptide was calculated using the binomial coefficients (1:8:28:56:70:56:28:8:1) as the weighting factors for the nine residues of the nonapeptide. Five phantom residues of zero hydrophobicity were appended to each end of the sequence so that the hydrophobicity plots did not start or end abruptly. Although the hydrophobicity of each of the nine residues of the nonapeptide contributes to each of the calculated points, the major contribution is from the central three residues. These smoothed curves were calculated and plotted using a Hewlett-Packard Model 9830A calculator fitted with a Model 9862A plotter.

RESULTS

Preliminary results showed that whole casein, of which α_{s1}-, α_{s2}-, β- and κ-casein are the major components, adsorbed onto Octyl- or Phenyl-Sepharose CL-4B from dilute phosphate buffers and could be totally desorbed with 6 *M* urea or 40% (v/v) ethanol. A gradient of 0–40% (v/v) ethylene glycol only desorbed a portion of the protein while the remainder was desorbed with 6 *M* solution. In every case, β-casein was eluted more readily than α_{s1}-casein.

Optimum separation was obtained when an increasing gradient of urea concentration was used in 0.1 *M* phosphate buffer. The maximum quantity of casein that could be chromatographed with good resolution of its components was about 0.25 g (3 mg/ml of Octyl-Sepharose). Thus large columns would be required if hydrophobic interaction chromatography were to be used to prepare large quantities of casein components.

Fig. 1 shows a typical chromatogram of whole casein containing equal quantities of the A and B variants of α_{s1}-casein. The initial peak of 280 nm absorbance which eluted at the void volume did not contain any protein as determined by gel

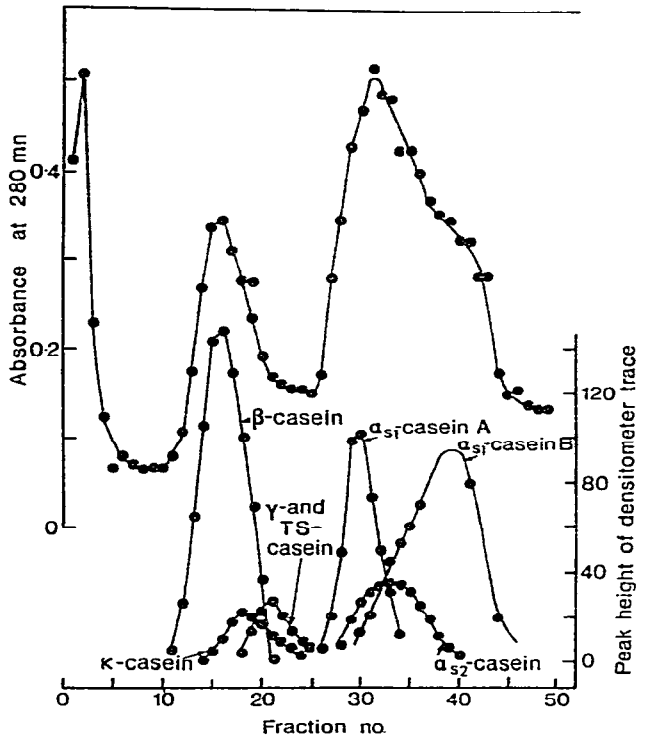

Fig. 1. Chromatography of whole casein on Octyl-Sepharose CL-4B. Upper curve: the absorbance at 280 nm *versus* fraction number is shown; lower curve: the densitometer peak heights for each component on disc gels are shown.

electrophoresis. The first major peak eluting near fraction 16 (320 ml of buffer, 120 ml into the urea gradient) was found by gel electrophoresis to be predominantly β-casein. The second larger peak of 280 nm absorbance was found to be a mixture of α_s-caseins by gel electrophoresis, α_{s1}-caseins A and B being well resolved from one another. When purified α_{s1}- and κ-caseins were chromatographed individually, they were eluted as symmetrical peaks at the peak positions (centered on fractions 40 and 18, respectively) expected on the basis of chromatography of whole casein. Changing the temperature of the system from 20–22°C to 1 or 40°C did not alter the order of elution of the components from the column, although all components eluted at higher urea concentrations at the higher temperature.

The pH of the system at 1°C determined both the order of elution and the overall tenacity of retention. In general the casein components were retained more strongly at the lower pH values (Fig. 2). The concentrations of Na^+, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, etc., that were present in the buffer changed with pH, and the elution positions may have been influenced by the modified concentrations of these ionic buffer components.

Chromatography of chymosin-treated casein

The elution pattern from a sample of whole casein that had been partially

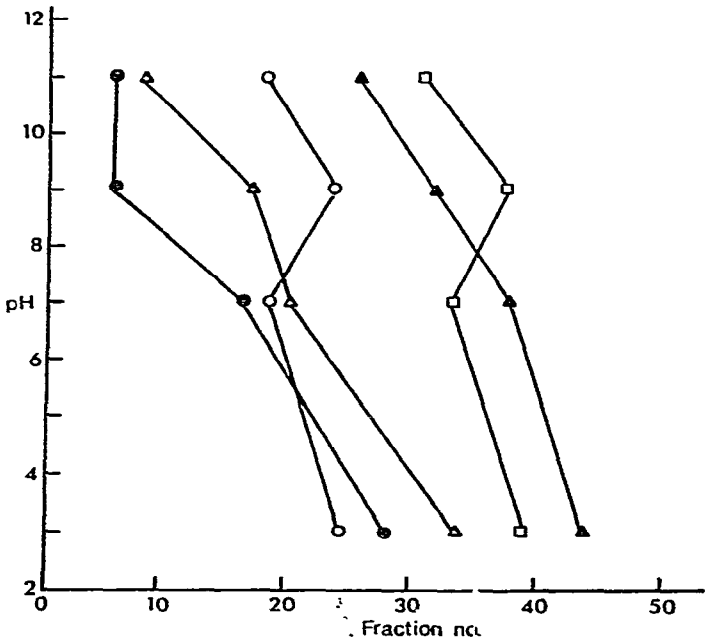

Fig. 2. Effect of pH on the chromatographic elution position of the casein components of whole casein. ●, β-Casein; △, γ-casein; ○, κ-casein; ▲, α_{s1}-casein; □, α_{s2}-casein.

degraded with chymosin is shown in Fig. 3. It is clear that the elution order is β-I, β-casein, para-κ-casein, α_{s1}-I, α_{s2}-casein and α_{s1}-casein.

An attempt to determine the elution position of para-κ-casein in isolation was not successful because it could not be dissolved and adsorbed to the column in the absence of α_{s1}- or β-casein.

Calculated hydrophobicities

Table I shows the calculated average hydrophobicities for a selection of casein components and derived peptides, while Fig. 4 shows the smoothed distribution of hydrophobicity along sequences of these proteins and peptides. It can be seen that the hydrophobicity of each casein or casein peptide is not evenly spread along its sequence and regions of higher-than-average hydrophobicity show as peaks in the curves. (The horizontal lines denote the average hydrophobicity of each protein or peptide.)

Varying the parameters in the smoothing calculation did not alter the shape of the curves greatly. However, smoothed curves are easier to comprehend than the jagged plots that are obtained when the hydrophobicity of each residue is plotted *versus* residue number.

Comparison of the hydrophobicity plots (Fig. 4) of α_{s1}-casein A and α_{s1}-I with that of α_{s1}-casein B shows that a peak of hydrophobicity near residue 25 of α_{s1}-casein B has diminished and that a small peak near residue 5 of α_{s1}-casein B is not present in α_{s1}-I. Overall, α_{s1}-casein B has only a few peaks of hydrophobicity, while β-casein A[2]

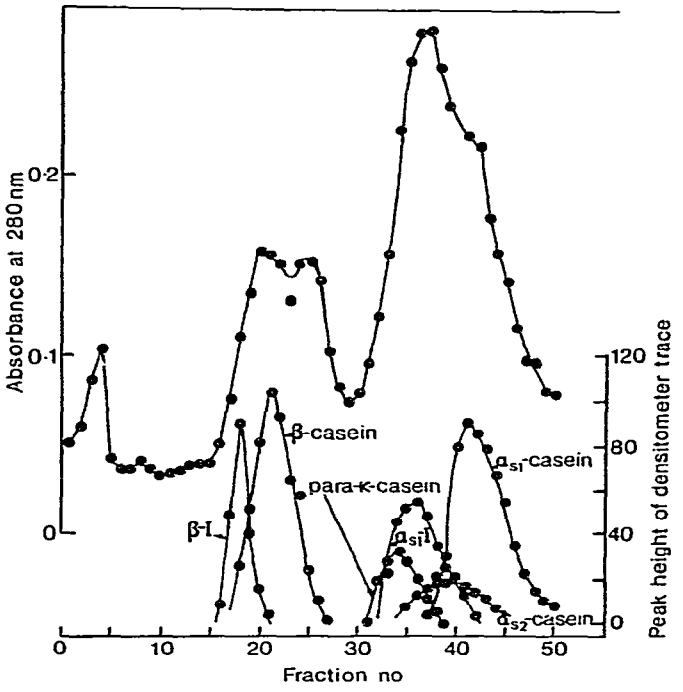

Fig. 3. Chromatography of chymosin-treated whole casein on Octyl-Sepharose CL-4B. The upper and lower curves are 280 nm absorbance and densitometer readings, respectively. However, the γ- and TS-casein curves are not shown and the para-κ-casein curve was derived from SDS disc gel densitometry.

TABLE I

AVERAGE HYDROPHOBICITIES OF BOVINE CASEINS AND CASEIN PEPTIDES

Protein or peptide	*Relationship to parent casein*	$H\varphi_{av}$* *(kJ/mole/residue)*
α_{s1}-Casein B		4.90
α_{s1}-Casein A	α_{s1}-Casein B (1–13, 27–199)	4.85
α_{s1}-I	α_{s1}-Casein B (25–199)	4.81
β-Casein A[2]		5.59
β-I	β-Casein A[2] (1–189)	5.35

* After Bigelow[10].

has a large number of peaks between residues 60 and 209. One of these peaks is absent from β-I, but none is absent from γ-casein (residues 29–209 of β-casein).

DISCUSSION

The present results show that the large casein peptides α_{s1}-I and β-I can be separated from their precursor proteins, α_{s1}- and β-casein. The rare A variant of α_{s1}-

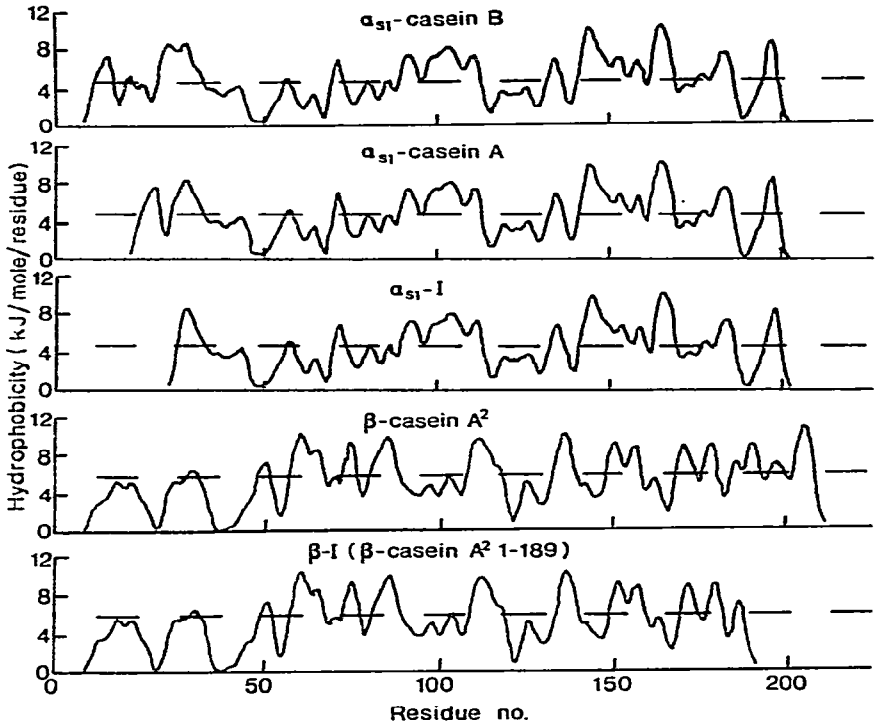

Fig. 4. Plots of the smoothed hydrophobicities of α_{s1}- and β-caseins. The calculations were carried out as described in the text. The horizontal lines are the average hydrophobicity for each peptide or protein. In some cases the plots are displaced to align the C-terminal residues.

casein is also readily separated from the common B variant, with which it often occurs in equimolar quantities. None of these separations is easily achieved using the standard methods of gel filtration[12] or cellulose ion-exchange chromatography[13,14]. Apart from these specific applications, the relatively low resolving power and capacity of the gels make their widespread use for routine isolation of casein components from whole casein less attractive.

The present results suggest a mechanism for the well-known method of preparing casein components[15] in which whole casein at its isoelectric point (approx. pH 4.6) is dissolved in 6 *M* urea and differentially precipitated as α_s- and β-casein by the step-wise addition of water; *i.e.*, dilution of the urea. Interpolation of the data shown in Fig. 2 to pH 4.6 indicates that α_{s1}-casein would interact more readily with hydrophobic groups than β- or κ-casein, and the self-interaction of α_{s1}-casein is thus likely to be hydrophobic. The present results also suggest that a similar mechanism may exist for the Zittle and Custer[16] method of preparing κ-casein by the acidification of whole casein dissolved in urea solution to precipitate α_s- and β-casein.

The most unexpected result from the present study was the early elution of β-casein compared with α_{s1}-casein when their calculated hydrophobicities (Table I) were in the opposite direction.

Kashavarz and Nakai[17] found that a series of globular proteins were not eluted

from a column of hexyloxy-Sepharose with an ethylene glycol gradient in the order of increasing average hydrophobicity, but that the order was closely correlated to the order of the distribution coefficients of those proteins between two immiscible phases of differing polarity. They concluded that both the distribution coefficients and the order of elution in hydrophobic interaction chromatography were governed by the size and structure of the surface "patches" of hydrophobic residues on the protein molecules. The caseins cannot be considered as globular proteins because they have a low degree of order in their structure[1,2,18,19], and yet their structures must be such that α_{s1}-casein has a region or regions that can interact strongly with the octyl groups attached to the Sepharose, while β-casein has no such region. Examination of the smoothed hydrophobicity *versus* residue number plots (Fig. 4) does not suggest that there is any difference between these proteins in their distribution and/or clustering of hydrophobic residues that might account for their observed elution behavior. It seems likely that the small amount of secondary structure that probably exists[18] in α_{s1}-casein is such that one or more regions of the molecule adopt a conformation that can react strongly with the octyl groups of the Octyl-Sepharose. The elution of both α_{s1}-casein A and α_{s1}-I prior to α_{s1}-casein B suggests that one such region may involve residues that are close to the sequence that is missing from both α_{s1}-casein A and α_{s1}-I, *viz.*, residues 14–24 of α_{s1}-caseins.

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