# Affinity Chromatography of Ovine Casein

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## ABSTRACT

Sheep milk casein was separated into two fractions: one containing $\alpha_{s1}$-plus β- and the other $\alpha_{s2}$ plus κ-caseins by affinity chromatography on activated thiol-Sepharose 4B. Milk samples were from the Leccese breed with the most common electrophoretic pattern. Electrophoresis of the chromatographic fractions on SDS-PAGE and on starch urea gel at pH 8.6 and 1.7 clarified the electrophoretic pattern of whole casein. Acidic pH electrophoresis of the two fractions obtained by affinity chromatography may be useful for investigations on the polymorphism of the casein fractions.
(Key words: ovine casein, affinity chromatography)

## INTRODUCTION

Research has not, so far, defined the components of sheep casein completely, and even less is known about $\alpha_s$-casein complex than about the κ- and β-caseins. Using ion-exchange chromatography of a composite casein from six Romney-Border Leicester crossbred ewes, Richardson and Creamer (23) have isolated $\alpha_s$-casein components and called them $\alpha_{s1}$-, $\alpha_{s2}$-, and $\alpha_{s3}$- according to their electrophoretic mobility on starch urea gel at pH 8.6. Recently Davoli et al. (13) observed that sheep milk contains two distinct fractions of $\alpha_s$-casein that can be separated into two distinct zones only by electrophoresis on starch urea gel at acidic pH but not alkaline pH. The two zones can probably be attributed to fractions $\alpha_{s2}$- and $\alpha_{s1}$-, the existence of which was demonstrated by Mercier et al. (19) by the preparation of cDNA from mRNA isolated from a sheep udder.

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Analytical techniques used so far for isolating sheep milk casein have been fractionation by chemical precipitation (1, 16, 29) and ion-exchange chromatography (1, 13, 20, 23, 24, 29). The affinity chromatography has not been used, to date, for the fractionation of ovine casein, but it has been used in the bovine (21), porcine (7, 8), and caprine (22) species. By a type of affinity chromatography, caseins without sulfhydryl (SH) groups ($\alpha_{s1}$- and β-) may be separated from the casein fractions containing SH groups of the amino acid residues cysteine and cystine (κ- and $\alpha_{s2}$-).

The objective of this research is to apply affinity chromatography in the study of sheep milk casein to separate the different fractions and establish their electrophoretic mobility.

## MATERIALS AND METHODS

### Sampling

Two individual skim milk samples from Leccese Italian sheep were used for the preparation of casein by isoelectric precipitation at pH 4.6. Casein was subsequently washed with water, ethanol, acetone, and ethyl ether.

### Chromatography

Casein (.500 g) was solved in 25 ml of .1 *M* Tris-HCl buffer, at pH 7.0, containing 7*M* urea, .3 *M* NaCl, 1 m*M* EDTA, and treated with .200 g of 1,4 dithiothreitol (DTT) to reduce disulfide bridges. The reduced casein (25 ml) was passed through a column of Sephadex G-25 (2.6 × 66 cm) by gel filtration to remove DTT. Successively, 100 ml of the eluted proteins were subjected to affinity chromatography (21) through a column containing 10 g of activated thiol-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The gel of the column (1.6 × 20 cm), containing 40 μ*M* 2-pyridyl disulfide groups, was equilibrated in Tris-HCl buffer at

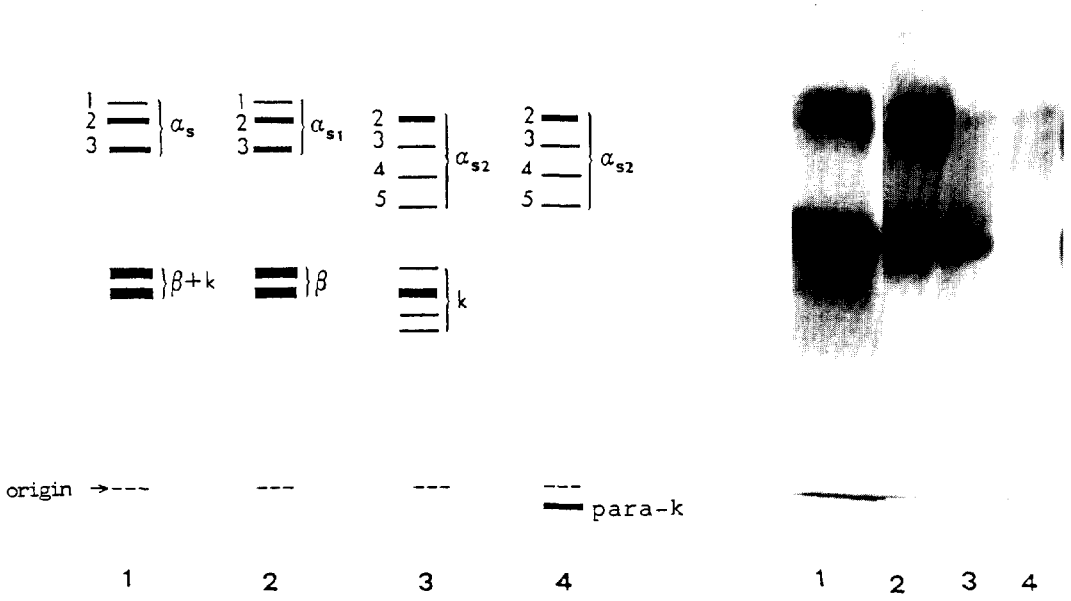


Figure 1. Electrophoretic patterns on starch urea gel at pH 8.6 of common casein (1) together with the fractions I and II isolated by affinity chromatography (2, 3) and II treated with rennin (4).

pH 7.0. The same buffer, which had been passed through a membrane filter (Millipore HVLP type, Bedford, MA) of pore size .45 μm, was also used as eluent for gel filtration and for the elution (240 ml) of the proteins not bound on activated thiol-Sepharose 4B. The covalently bound proteins were eluted with 60 ml of Tris-HCl buffer at pH 8.0 containing 25 m*M* cysteine. The flow-rate was 10 ml/h. The regeneration of the gel activated thiol-Sepharose 4B was performed with 1.5 m*M* 2-2-dipyridildisulfide in buffer Tris-HCl at pH 8.0.

### Gel Electrophoresis

The two fractions obtained were dialyzed against polyethylene glycol and characterized by electrophoresis on starch gel (SGE) in urea at pH 8.6 (4) and 1.7 (3) and on SDS-PAGE by the discontinuous system (15, 18) together with the corresponding whole casein.

### Treatment with Rennin

In order to study the κ-casein, the fractions obtained by affinity chromatography, adjusted to pH 6.6 by .076 *M* Tris-citrate buffer, were treated with rennin (chymosin; EC 3.4.23.4). The rennin was dispersed in .076 *M* Tris-citrate buffer at pH 6.6 (55 units/ml), and 30 μl were added to 500 μl of the fractions at 37°C for 30 min. Subsequently, urea at a final concentration of 8 *M* was added to inactivate it. The treated fractions were used for electrophoresis (3, 4, 15, 18).

## RESULTS AND DISCUSSION

The samples used for the analyses were characterized by the electrophoretic pattern indicated as the most common in literature (2, 9, 10, 11, 12, 13, 14, 17, 23, 25, 27, 28). This pattern at alkaline pH presents two zones of bands (Figure 1): the faster one with three bands is called $\alpha_s$-casein (2, 9, 10, 11, 12, 13, 14, 17, 25, 27) or $\alpha_{s1}$-, $\alpha_{s2}$-, and $\alpha_{s3}$-, according to their electrophoretic mobility (23). The other, slower zone presents two bands attributed to β-casein (24) and κ-casein (1, 16, 20, 29). At acidic pH, three migration zones can be identified (Figure 2). The fastest zone comprises two bands, the intermediate three, and

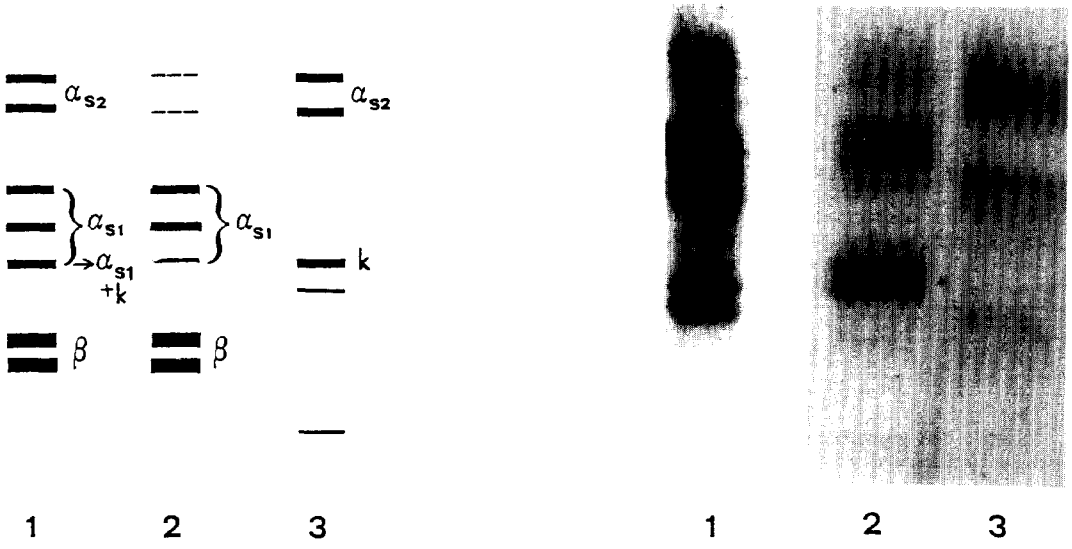


Figure 2. Eletrophoretic patterns on starch urea gel at pH 1.7 of common casein (1) together with the fractions I and II isolated by affinity chromatography (2, 3).

the slowest two. Davoli et al. (13) have hypothesized that these zones can be attributed, in order of decreasing mobility, to the $\alpha_{s2}$-, $\alpha_{s1}$- and β-caseins, in keeping with observations made in goat studies (5, 26).

Affinity chromatography allows the separation of sheep casein into two fractions. The first (fraction I) comprises proteins that are unbound to the column since they do not contain cystine and cysteine residues and are thus represented by β-casein (24) and $\alpha_{s1}$-casein (23). The second (fraction II), eluted by adding a reducing agent to the buffer, comprised proteins covalently bound to the gel by thiol groups and, thus, by κ-casein (1, 16, 29) and $\alpha_{s2}$-casein. The amino acid composition of the latter has not yet been defined, but this fraction is likely to contain cysteine residues in keeping with that of other species (5, 6).

The electrophoretic patterns on SGE at pH 8.6 and 1.7 and on SDS-PAGE of fractions I and II obtained by affinity chromatography are reported in Figures 1, 2, and 3 together with the corresponding whole casein.

In SDS-PAGE, the whole ovine casein pattern shows four bands (Figure 3). In the fraction II containing κ- and $\alpha_{s2}$-caseins are visible three bands. Electrophoresis of this fraction treated with rennin was performed in order to distinguish κ-casein. As a result, the two faster bands could be attributed to κ-casein. Indeed, following treatment, these bands disappear from the electrophoretic pattern with the appearance of a faster band corresponding to the para-κ-casein. The remaining band of minor electrophoretic mobility of the retained fraction, can be attributed to the $\alpha_{s2}$-casein.

The fraction I on SDS-PAGE exhibits one band for both β-casein and $\alpha_{s1}$-casein (Figure 3). The four bands visible in the whole casein pattern on SDS-PAGE can thus be attributed, in order of decreasing mobility, to the κ-, β- + κ-, $\alpha_{s1}$-, and $\alpha_{s2}$-caseins. Therefore, SDS-PAGE gives complete separation of $\alpha_{s1}$- and $\alpha_{s2}$-caseins, but it is not useful for identification of the variants of the single casein fractions with similar molecolar weights (12).

The SGE of fraction II at alkaline pH shows several bands (Figure 1). As a result of electrophoresis of fraction II treated with rennin, the band with same mobility as the slowest β-casein level and three other weak bands migrating in the same zone could be attributed to κ-casein. After treatment with rennin, these bands disappear from the electrophoretic pattern with the appearance of a band of cathodic speed

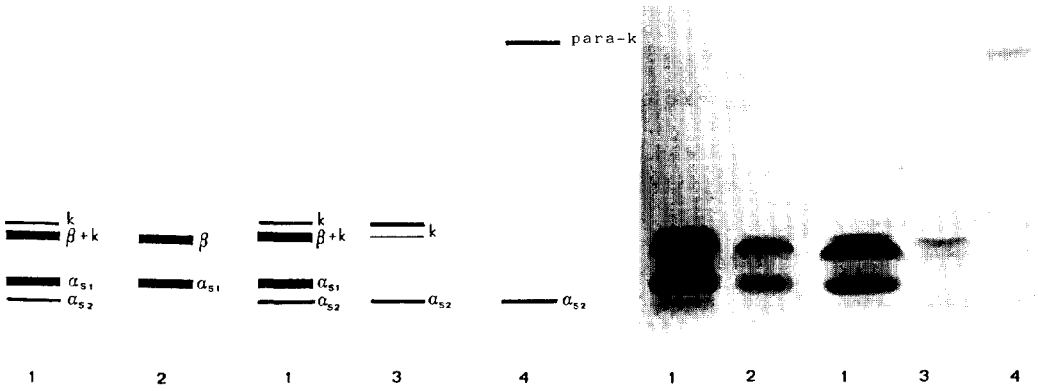


Figure 3. Electrophoretic patterns on SDS-polyacrylamide gel of common casein (1) together with the fractions I and II isolated by affinity chromatography (2, 3) and II treated with rennin (4).

corresponding to the para-κ-casein. The principal κ-casein band has the same mobility as the β-casein and, thus, is not distinguished from it in the analyses of the whole casein. The remaining bands of highest electrophoretic mobility of the retained fraction, which migrate to levels 2, 3, 4, and 5 can be attributed to the $\alpha_{s2}$-casein. The $\alpha_{s2}$-casein bands are not very intense and only show up if concentrated. This explains why at the concentration used in the electrophoretic analysis of the whole casein, the $\alpha_{s2}$-casein bands at levels 4 and 5 are not visible.

Overall the data indicate that electrophoresis at pH 8.6 is not suitable for studying sheep milk caseins.

Electrophoresis at pH 1.7 of fraction II (Figure 2) shows three main bands. The slowest, which migrates in the intermediate zone, belongs to the κ-casein since electrophoresis after treatment with rennin showed the disappearance of this band and the appearance of a para-κ-casein band. The two most mobile bands, which migrate to the same level as the fastest band of the whole casein pattern, belong to $\alpha_{s2}$-casein. At pH 1.7, the four $\alpha_{s2}$-casein bands observed at pH 8.6 give rise to two equally intense bands. In fraction II, other weak bands also are evident (Figure 2). These bands are κ-casein, due to their sensitivity to rennin.

Electrophoresis of fraction I shows the two β-casein bands and the $\alpha_{s1}$-casein. The $\alpha_{s1}$-casein gives rise to three bands both at alkaline and acidic pH. At pH 8.6, these bands migrate to the three levels of the $\alpha_s$-casein zone visible in the pattern of the whole casein. It is true to say that in the whole casein pattern at alkaline pH, the band at levels 2 and 3 of $\alpha_{s1}$-casein overlap the $\alpha_{s2}$-casein bands, which migrate to the same levels. At acidic pH, the $\alpha_{s1}$-casein migrates to the intermediate mobility zone of the whole casein pattern. Therein are the three bands Davoli et al. (13) have attributed to that casein fraction. In the unbound fraction, residual bands of κ- and $\alpha_{s2}$-caseins also are visible. The results of electrophoresis at pH 1.7 of fraction I treated with rennin to obtain hydrolysis of κ-casein are confirmed (13) that at this pH, the least mobile band of $\alpha_{s1}$-casein migrates to the level of the principal band of κ-casein (Figure 2).

## CONCLUSIONS

Affinity chromatography enabled a separation of the ovine $\alpha_s$-casein into two fractions, $\alpha_1$-, and $\alpha_{s2}$-. As a result, it was possible to establish the electrophoretic image of the most common pattern of the whole casein on SGE at alkaline and acidic pH and on SDS-PAGE.

The electrophoresis of the whole casein at alkaline pH shows an overlapping of the bands of the casein $\alpha_{s1}$- and $\alpha_{s2}$- and those of β- and κ. The electrophoresis at acidic pH gives complete separation of $\alpha_{s1}$- and $\alpha_{s2}$-caseins,

whereas the main band of κ-casein overlaps the slowest band of $\alpha_{s1}$-casein.

Electrophoresis at acidic pH of the fractions obtained by affinity chromatography, despite the lengthy execution time required, may be useful for investigations on the polymorphism of these protein fractions.

## ACKNOWLEDGMENTS

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