



Human α_{s1} -casein: purification and characterization

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The human counterpart of α_{s1} -casein has been purified by a combination of gel-filtration and ion-exchange chromatography under denaturing conditions. SDS-PAGE analysis revealed the presence of a diffuse ladder with a high molecular mass which upon reduction was replaced by several closely spaced bands of lower molecular masses and a broad diffuse band corresponding to κ -casein. Amino acid sequence analysis of the closely spaced bands all resulted in the same N-terminal sequence which was found to be homologous with α_{s1} -casein from other species. Sequence analysis of a major radiolabelled tryptic peptide from purified ^{14}C -carboxymethylated α_{s1} -casein demonstrated that the protein contains at least two cysteine residues. As judged by SDS-PAGE in the presence or absence of a reducing agent, the molecular structure of the polymers constituting the ladder is composed of heteropolymers of α_{s1} - and κ -casein cross-linked by disulfide bonds.

Key words: Disulfide bridges; Heteromultimer; Human α_{s1} -casein; Human milk; κ -casein; Micellar structure; N-terminal sequence; Purification.

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Introduction

The caseins are the predominant milk proteins of almost all mammalian species (Jenness and Holt, 1987). They constitute a heterogeneous group of phosphoproteins present as stable calcium phosphate protein complexes termed micelles. The biological functions of the caseins are to provide the progeny with a source of phosphate and calcium for the mineralization process of calcified tissues, as well as amino acids and biologically active peptides (for reviews see Migliore-Samour and Jollès, 1988; Miller *et al.*, 1990). The caseins are one of the most rapidly evolving families of proteins (Mercier *et al.*, 1976; Yu-Lee *et al.*, 1986) and have been shown to be members of a single multigene family in at least three species (Gupta *et al.*, 1982; Dalens and Gellin, 1986; Ferretti *et al.*, 1990; Threadgill and Womack, 1990). Ruminant caseins, which have been extensively

studied, comprise four components; α_{s1} -, α_{s2} -, β - and κ -casein (for reviews see Swaisgood, 1992; Mercier and Vilotte, 1993). The α_s - and β -caseins are insoluble in the presence of calcium ions encountered in milk while κ -casein is insensitive to calcium and plays a key role in maintaining the stability and integrity of the micelle structure. In the bovine system, κ -casein has been shown to exist as homomultimers linked by disulfide bridges in a random pattern (Rasmussen *et al.*, 1992).

Although milk protein composition can differ quantitatively and/or qualitatively from one species to another, all species examined so far at the molecular level contain α -, β - and κ -casein. Missing in this scenario is human milk where it is more or less generally accepted that the casein micelle is composed of only β - and κ -casein while the α -casein subunit is absent or found in very small amounts (Kunz and Lönnerdal, 1990). Both proteins have been characterized at the protein level (Greenberg *et al.*, 1984; Brignon *et al.*, 1985) and at the cDNA level (Menon and Ham, 1989; Bergström *et al.*, 1992). However, earlier studies including two-

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dimensional gel electrophoresis have indicated the presence of an α -casein component as well (Voglino and Ponzone, 1972; Anderson *et al.*, 1982) and recently, a N-terminal sequence from a human α_{s1} -casein like protein has been published (Cavaletto *et al.*, 1994).

Here we report the presence of the human counterpart of α_{s1} -casein, its purification, N-terminal sequence and its multimeric nature.

Materials and Methods

Reagents

Sephacrose CL-6B, Superdex 200 HR 10/30 and Mono S HR 5/5 were obtained from Pharmacia (Uppsala, Sweden). Iodo[2- ^{14} C]acetic acid (52 mCi mmol $^{-1}$) was from Amersham, UK. The molecular mass standards for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). ProBlott membranes and reagents used for sequencing were purchased from Applied Biosystems (Foster City, CA). All other chemicals were of analytical grade.

Preparation of human casein

Human milk was provided by two healthy mothers 1–2 months after parturition. Skimmed milk was prepared from the individual milk samples by centrifugation at 2600 g at 4°C for 10 min. Casein micelles were obtained from skimmed milk by ultracentrifugation (120 000 g) at 4°C for 1.5 hr.

Gel electrophoresis

SDS-PAGE was performed according to Laemmli (1970) on 10–20% (w/v) gradient gels which were stained with Coomassie Blue R-250.

Amino acid sequence analysis

Automated Edman degradation was carried out on an ABI 477A/120A protein sequencer (Applied Biosystems) using standard programs. Half-cysteine was determined as carboxymethyl-cysteine.

Gel-filtration chromatography

Human casein was separated on a Sepharose CL-6B column (4 × 95 cm) eluted with ammonium acetate/urea buffer (50 mM CH₃COONH₄, 8 M urea, pH 5) at a flow rate of 35 ml hr $^{-1}$. The effluent was monitored at 280 nm and collected in fractions of 3 ml.

Purification of ^{14} C-carboxymethylated α_{s1} -casein

Pool B from the gel-filtration chromatography was reduced and carboxymethylated. Lyophilized material (4 mg) was dissolved in 1 ml 8 M urea, 0.3 M Tris, pH 8.3 and incubated with 6 μ mol dithioerythritol for 0.5 hr in the dark at 20°C with stirring. Iodo[^{14} C]acetic acid

(12.5 μ Ci) was added and after 15 min the mixture was supplemented with 15 μ l iodoacetic acid (final concentration 28 mM). The reaction was allowed to proceed for an additional 15 min after which it was stopped, desalted and freeze-dried. The material was applied to a Superdex 200 column eluted with ammonium acetate/urea buffer (50 mM CH₃COONH₄, 8 M urea, pH 6) followed by separation on a Mono S column equilibrated with the same buffer and eluted with a gradient of 1 M ammonium acetate, 8 M urea, pH 6. The separations were carried out at a flow rate of 0.5 ml min $^{-1}$ on a Pharmacia LKB HPLC system consisting of a 2248 LC gradient pump connected to a 2252 LC controller and a 2510 Uvicord detector equipped with a 278 nm filter and a flow cell with a 2.5 mm path length.

Results and Discussion

Human casein micelles obtained by ultracentrifugation were separated by gel-filtration chromatography (Fig. 1) and the various pools were analysed by SDS-PAGE (Fig. 2). Pool B showed a number of bands forming a diffuse ladder of higher molecular mass (panel B, lane 1). In the presence of dithioerythritol these bands, except one, were replaced by several bands (panel B, lane 2); (i) a broad diffuse band in the range of 40–55 kDa and (ii) at least four closely spaced bands with molecular masses between 25–32 kDa. Pool C also showed a ladder of bands (panel B, lane 3) but with a lower molecular mass, and after reduction a similar pattern of bands was discerned (panel B, lane 4). Furthermore, pool C contained a protein with a molecular mass of approximately 66 kDa which upon reduction migrated with a molecular mass of 75 kDa. In order to establish the identities of the various proteins, pools A–E in the absence or presence of a reducing agent

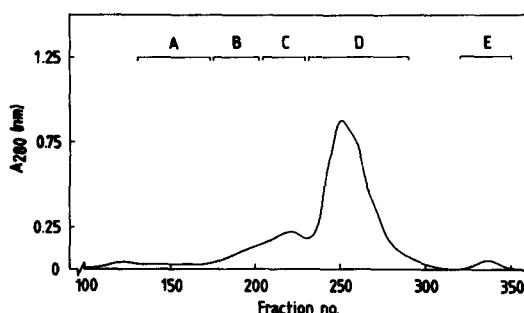


Fig. 1. Gel-filtration of casein from human milk. The proteins were separated on a Sepharose CL-6B column eluted with ammonium acetate/urea buffer (50 mM CH₃COONH₄, 8 M urea, pH 5) at a flow rate of 35 ml hr $^{-1}$. Proteins were detected in the effluent by recording the absorbance at 280 nm (—). Fractions were pooled (A–E) as indicated.

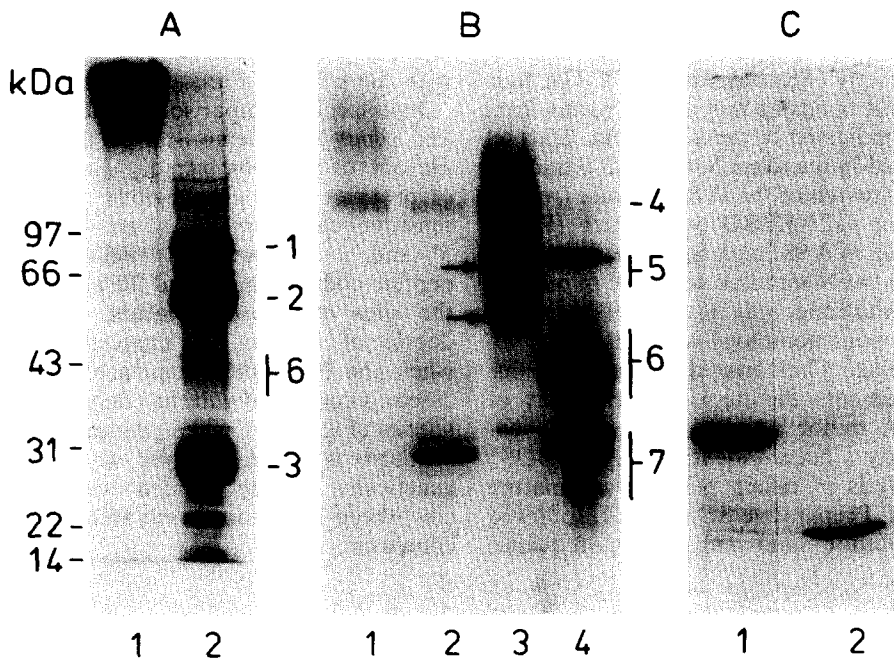


Fig. 2. SDS-PAGE analysis of human casein. Pools (A–E) from gel-filtration (Fig. 1) were analysed on a 10–20% linear gradient polyacrylamide gel and stained with Coomassie Blue R-250. Panel A shows pool A in the absence (lane 1) and presence of dithioerythritol (lane 2). Panel B shows pools B (lanes 1, 2) and C (lanes 3, 4) in the absence and presence of dithioerythritol. The arrows indicate the bands from the ladder which were sequenced (see text). Numbered bands (1–7), indicated to the right, correspond to secretory component, IgG heavy chain, immunoglobulin light chain and monomeric α_{s1} -casein, bile-salt stimulated lipase, lactotransferrin, monomeric κ -casein, and monomeric α_{s1} -casein, respectively, as determined by amino acid sequence analysis of electroblotted bands (see text). Panel C shows pools D (lane 1) and E (lane 2) corresponding to β -casein and lysozyme, respectively. Molecular mass markers are indicated to the left.

	1	14
<i>Human</i>	R P K L P L R Y P E R L Q N	
<i>Swine</i>	R P K P P L R H Q E H L Q N	
<i>Guinea pig</i>	M P K F P F R H T E L F Q T	
<i>Cow</i>	R P K H P I K H Q G L P Q E	
<i>Goat</i>	R P K H P I N H Q G L S P E	
<i>Sheep</i>	R P K H P I K H Q G L S P E	
<i>Rabbit</i>	R H K F H L G H L K L T Q E	
<i>Mouse</i>	M P R L H S R N A V S S Q T	
<i>Rat</i>	L P R A H R R N A V S S Q T	
<i>Wallaby</i>	R P D A L R L S I D R H F K	

Fig. 3. Comparison of the N-terminal sequence of human α_{s1} -casein with its counterpart in other species. The amino acid sequence of bovine α_{s1} -casein is according to Mercier *et al.* (1971). The amino acid sequences deduced from the cDNA sequences of goat, sheep, guinea pig, mouse, rat, wallaby, rabbit and swine are according to Brignon *et al.* (1989), Mercier *et al.* (1985), Hall *et al.* (1984), Grusby *et al.* (1990), Hobbs and Rosen (1982), Collet *et al.* (1992), Devinoy *et al.* (1988) and Alexander and Beattie (1992), respectively. Residues identical with the human sequence are shown in bold in each species.

were subjected to SDS-PAGE analysis, electroblotted on to a Problott membrane, and sequenced directly (Matsudaira, 1987). The four closely spaced bands as well as two bands from the ladder (indicated by arrows in Fig. 2, panel B) all resulted in the same N-terminal sequence. A computer search of the PIR database (release 37) using the GENEPRO programme (Riverside Scientific, WA 98110, USA) revealed a clear similarity to the N-terminal sequence of porcine α_{s1} -casein (Alexander and Beattie, 1992). Moreover, no obvious homology was identified with other proteins. Thus, we conclude that this protein represents the human counterpart of α_{s1} -casein. A minor α_{s1} -casein like component has recently been identified by SDS-PAGE analysis of casein pellets from mature human milk. Preparative SDS-PAGE followed by electroelution and subsequent sequence

analysis gave a N-terminal sequence (Cavaletto *et al.*, 1994). Our obtained sequence is consistent with the published sequence.

Interspecies comparisons of cDNA sequences and genes have demonstrated that the caseins are one of the most rapidly diverging families of proteins. Only few regions of the calcium-sensitive casein mRNAs are conserved: the 5' and 3' untranslated regions, the signal peptide-coding region, and the regions encoding the sites of phosphorylation. Hence, a low degree of identity, as expected, is observed when the N-terminal sequence of human α_{s1} -casein is compared with its counterpart in other species (Fig. 3). A more pronounced sequence similarity is found, however, when more phylogenetically closely related species such as cow-sheep-goat, mouse-rat and pig-human are compared.

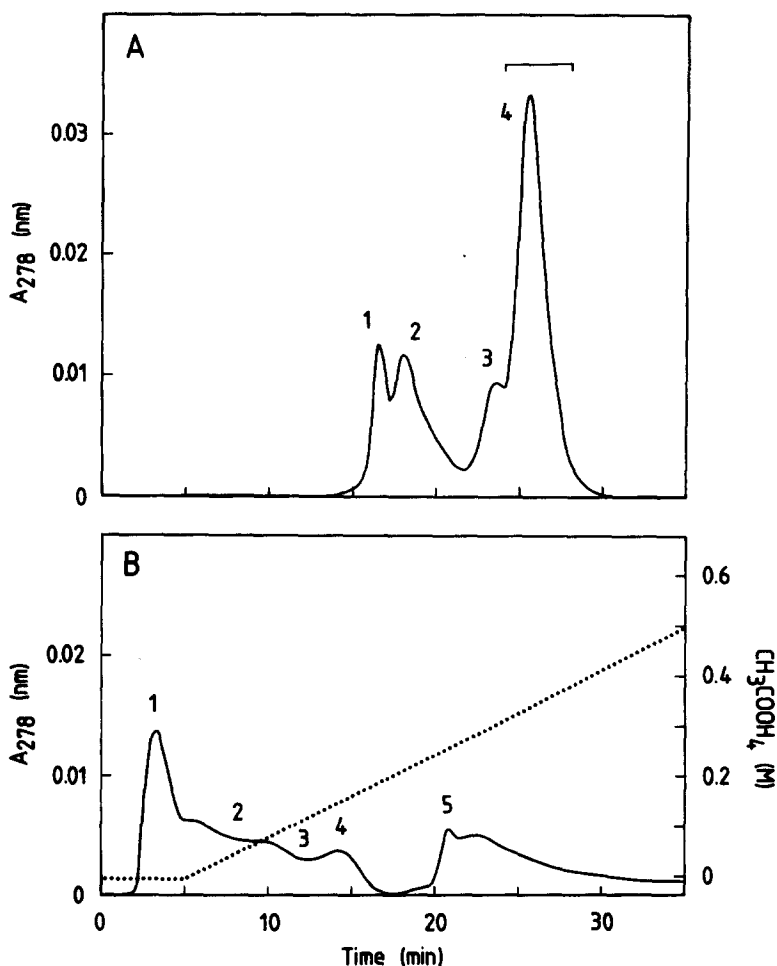


Fig. 4. Purification of ^{14}C -carboxymethylated α_{s1} -casein. (A) Pool B from gel-filtration (Fig. 1), reduced and carboxymethylated, was separated on a Superdex 200 column eluted with ammonium acetate/urea buffer (50 mM $\text{CH}_3\text{COONH}_4$, 8 M urea, pH 6) at a flow rate of 0.5 ml min^{-1} . (B) Peak 4, containing κ - and α_{s1} -casein (indicated by the bar in Fig. 4A) was further separated on a Mono S column eluted with a gradient of 1 M ammonium acetate, 8 M urea, pH 6 (...) at a flow rate of 0.5 ml min^{-1} . The effluents were monitored at 278 nm (—) and collected manually.

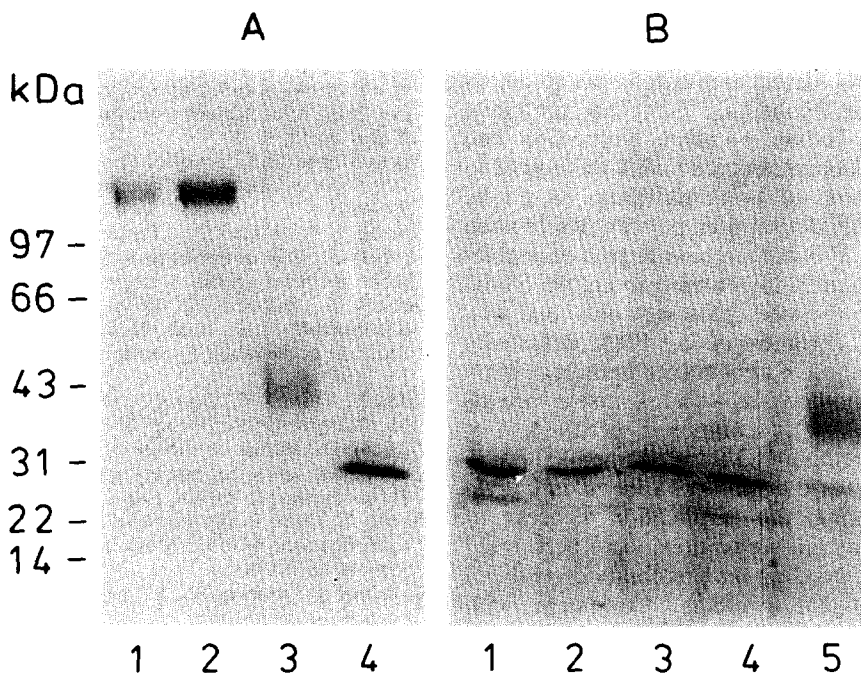


Fig. 5. SDS-PAGE analysis of ^{14}C -carboxymethylated α_{s1} -casein during purification. Fractions from gel-filtration and ion-exchange chromatography (Fig. 4A and B) were analysed on a 10–20% gradient polyacrylamide gel and stained with Coomassie Blue R-250. Lanes 1–4 in panel A and lanes 1–5 in panel B correspond to peaks 1–4 and 1–5 in Fig. 4A and B, respectively. Molecular mass markers are indicated to the left.

Amino-terminal sequence analyses showed that the bands appearing after reduction of pool A corresponded to secretory component (Eiffert *et al.*, 1984), immunoglobulin heavy chain, immunoglobulin light chain and α_{s1} -casein. As judged from the electrophoretic behaviour, the broad diffuse band only observed in the presence of dithioerythritol in both pools A, B and C corresponded to human κ -casein which is known to be heavily glycosylated (Brignon *et al.*, 1985). No sequence results could be obtained from this band consistent with a pyroglutamate as the N-terminal residue. The 150 kDa band seen in pools B and C corresponded to the human bile-salt stimulated lipase (Nilsson *et al.*, 1990) and the 66 kDa band was shown to be lactotransferrin (Metz-Boutigue *et al.*, 1984). Finally, pools D and E contained human β -casein (Greenberg *et al.*, 1984) and lysozyme (Jollès and Jollès, 1972), respectively. Both immunoglobulins, lysozyme, lipase and lactotransferrin are whey protein contaminants caused by an incomplete separation of whey proteins and whole casein by ultracentrifugation.

To obtain purified monomeric α_{s1} -casein subunits, pool B was first reduced with dithioerythritol followed by alkylation of free SH groups with radioactive iodoacetic acid. Monomeric α_{s1} -casein could then be purified by a combination of gel-filtration and ion-exchange chromatography (Fig. 4A and B). The human

bile-salt stimulated lipase (Fig. 4A, peak 1, 2) was well separated from κ - and α_{s1} -casein (Fig. 4A, peak 3, 4) by gel-filtration as shown by SDS-PAGE analysis (Fig. 5). Using ion-exchange chromatography as a second purification step, a complete separation of α_{s1} -casein (Fig. 4B, peaks 1–4) and κ -casein (Fig. 4B, peak 5) was obtained.

Earlier comparative gel electrophoretic studies of bovine and human caseins have shown the presence of several bands which based on their electrophoretic positions have been identified as the human homologue of α -casein (Vogliano and Ponzzone, 1972; Anderson *et al.*, 1982). In agreement with these findings, at least four bands corresponding to monomeric α_{s1} -casein were observed in SDS-PAGE under reducing conditions. However, whether these bands reflect heterogeneity in phosphorylation sites, genetic variants, multiple mRNA caused by alternative splicing or simply degradation products of α_{s1} -casein remain to be elucidated. Genetic polymorphism as well as variation in the degree of phosphorylation are common features of the calcium-sensitive caseins (Ng-Kwai-Hang, 1992). Likewise, exon skipping in the case of α_{s1} - and α_{s2} -casein genes resulting in multiple mRNA species is not an uncommon event (Mercier and Vilotte, 1993). Furthermore, the existence of two gene products has been reported for rabbit α_{s2} -casein (Dawson *et al.*, 1993).

A functional aspect of κ -casein is to stabilize the micelle structure in milk, and several studies indicate that κ -casein is located mainly at the micelle surface (Rollemma, 1992). The multimeric structure of bovine κ -casein, containing two cysteines, has been resolved and it was found to exist as a series of homomultimers linked by disulfide bonds in a random pattern (Rasmussen *et al.*, 1992). The human counterpart resembles bovine κ -casein in its multimeric nature. However, human κ -casein possesses only one cysteine residue (Brignon *et al.*, 1985; Bergström *et al.*, 1992) and the appearance upon reduction of both monomeric α_{s1} - and κ -casein reveals that the ladder is composed of these two proteins forming disulfide-linked heteropolymers in various sizes and in an unknown pattern. During the purification process of human caseins, all separations were performed below pH 6 excluding the possibility of artificial oxidation of the proteins. Consistent with the formation of disulfide-linked heteropolymers, sequence analysis of a major radiolabelled tryptic peptide from purified ^{14}C -carboxymethylated α_{s1} -casein (Cys-Ala-Glu-Gln-Phe-Cys-Arg) demonstrated that the protein contained at least two cysteine residues. A possible physiological function of the multimeric state of κ -casein in both the bovine and in the human system is to facilitate κ -casein in covering the micelle surface, thereby stabilizing the micellar structure (Rasmussen *et al.*, 1992).

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