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Characterization of a heterogeneous camel milk whey non-casein protein

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A milk protein, occurring in the whey fraction, has been characterized from camel milk. Determination of the primary structure reveals the existence of two related types of chain with residue differences in at least the N-terminal region. A fragment representing an N-terminal part of the protein was also recovered (heterogeneous at the same positions). The absence of cysteine residues in the protein shows that no disulphide bridges are present. The pattern of fragments and a parent protein resembles that for casein and its fragments, showing that fragments and a multiplicity of forms may be typical for different milk proteins.

Milk whey protein; Heterogeneity; Protein fragment; Amino acid sequence

1. INTRODUCTION

Milk whey contains proteins of different types which have been characterized from several species. Such proteins include α-lactalbumin, found in milk of all ruminants and non-ruminants so far studied (a recent addition in [1]); β-lactoglobulin, which appears to be mostly confined to ruminants and some non-ruminants, but its presence in some species is controversial [2,3]; a half-cystine rich protein characterized from rat, mouse [4,5] and camel [6,7] milk; and casein and its fragments. β-Casein, well studied in several species, is known to produce fragments [8,9]; some short β-casein segments, casomorphin and morphiceptin peptides, have been found to have opioid activity [10–13], but different casein cleavages have been demonstrated, and in camel a new type of fragment was recently characterized [14].

The variability of forms, structures and distributions of these proteins make further studies desirable. We have therefore decided to characterize camel (*Camelus dromedarius*) milk proteins, and have recently reported three of the structures mentioned above. We now report on the presence in the whey fraction of a protein lacking cysteine residues. The polypeptide chain was found to be present in two forms with different sizes, one corresponding to an N-terminal fragment of the longer form. Furthermore, the two chains were found to contain positional heterogeneities.

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2. MATERIALS AND METHODS

Fractionation of camel milk whey proteins by exclusion chromatography on Sephadex G-100 [6] gives four fractions (numbered I–IV in fig.1, [6]), which are purified by reverse-phase HPLC, using a gradient of acetonitrile in 0.1% trifluoroacetic acid [6]. Fractions were checked by N-terminal sequence analysis, SDS-polyacrylamide gel electrophoresis, and amino acid composition after hydrolysis. Protein samples (100 nmol) for analyses were cleaved with CNBr (200 mg) in 70% formic acid for 24 h at room temperature. For enzymatic cleavages, trypsin, staphylococcal ex-

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tracellular Glu-specific protease, *Achromobacter lyticus* Lys-specific protease, and clostripain were used in 0.2 M ammonium bicarbonate with enzyme to substrate ratios of 1:100 at pH 8.0, 37°C for 4 h. Peptides from all digests were fractionated by reverse-phase HPLC on a μBondapak C18 column in 0.1% trifluoroacetic acid containing a linear gradient of acetonitrile.

Pure peptides were analyzed by sequence degradations with the manual DABITC method [15,16], and for larger peptides, by liquid-phase sequencer degradations (on 10–20 nmol, with repetitive yields of ⩾95%) in the presence of glycine-precycled Polybrene [17]. Phenylthiohydantoin derivatives were identified by reverse-phase high-performance liquid chromatography [18]. Amino acid compositions were determined on a Beckman 121M analyzer after hydrolysis at 110°C for 24 h in 6 M HCl containing 0.5% phenol.

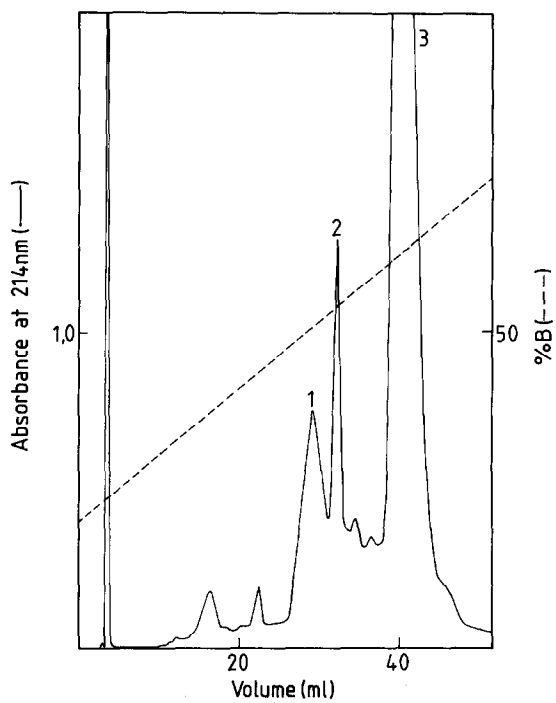

Fig.1. Final purification of the novel camel milk protein by reverse-phase high-performance liquid chromatography. Column: μBondapak C18, developed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Peaks: 1, serum albumin; 2, α-lactalbumin; 3, the protein presently described. The material applied was obtained from a step of exclusion chromatography on Sephadex G-100 (peak 2, fig.1 [6]).

3. RESULTS

3.1. *Isolation*

Camel milk whey proteins were separated into four fractions by Sephadex G-100 chromatography as described [6]. The whey protein lacking cysteine was further separated by reverse-phase HPLC (fig.1), giving a non-casein protein (peak 3, fig.1). In the Sephadex chromatography step, this material eluted at a volume corresponding to 30 kDa proteins. However, as judged by SDS-polyacrylamide gel electrophoresis, the final material gave a single diffuse band in the range of 15 kDa.

3.2. *Structural analysis*

The protein was cleaved with CNBr, trypsin,

Table 1

Total composition of the intact protein and its CNBr fragments

Residue	Intact protein	Peptide			
		CNBr1a	CNBr1b	CNBr2	CNBr3
Asx	11.0 (11)	2.0 (2)	1.0 (1)	8.6 (8)	1.2 (1)
Thr	9.5 (9)	– –	– –	8.0 (8)	– –
Ser	10.7 (10)	1.0 (1)	1.0 (1)	7.5 (7)	2.1 (2)
Glx	17.0 (17)	1.0 (1)	2.0 (2)	13.0 (13)	2.0 (2)
Pro	7.9 (8)	1.4 (1)	– –	5.0 (5)	1.8 (2)
Gly	2.0 (2)	– –	– –	2.2 (2)	– –
Ala	4.7 (5)	– –	2.0 (2)	4.0 (4)	0.9 (1)
Val	6.5 (7)	– –	0.5 (1)	5.0 (5)	1.5 (2)
Met	4.9 (4)	0.3 (1)	0.3 (1)	0.5 (1)	– –
Ile	3.8 (4)	0.5 (1)	0.5 (1)	2.6 (3)	– –
Leu	11.5 (12)	0.9 (1)	0.9 (1)	8.7 (9)	1.7 (2)
Tyr	0.7 (1)	– –	0.1 (1)	– –	– –
Phe	2.7 (3)	– –	– –	1.0 (1)	1.7 (2)
Lys	9.7 (10)	0.8 (1)	– –	7.1 (7)	2.5 (2)
His	4.6 (4)	– –	– –	3.0 (3)	1.3 (1)
Arg	4.3 (5)	– –	– –	3.4 (4)	– –
Sum	112	9	11	80	17

Results from acid hydrolysis for 24 h are given in molar ratios, together with values (within parentheses) from the sum of sequence analysis. Met was analyzed as homoserine in the hydrolysates of the CNBr fragments, explaining the low recoveries

Glu-specific protease, a Lys-specific protease, and for the large CNBr fragment, clostripain. The peptides were separated by reverse-phase HPLC on a μBondapak C18 column in the same way as the intact protein. Analysis of all fragments gave the amino acid sequence of two apparently largely identical chains as shown in fig.2. Except for the smallest CNBr fragment all the CNBr fragments were recovered (fig.3). Their compositions are given in table 1, and their sums agree with the values obtained from the whole protein. The compositions and sequences of all other peptides are also in agreement with the structure shown in fig.2. CNBr1 represents the N-terminal, heterogeneous part, while CNBr2 covers the middle part and CNBr3 is the C-terminal part of both subunits. The region between CNBr2 and CNBr3 could be determined by overlapping peptides from other digests. The N-terminus of the protein was identified as Ser by sequence analysis of intact protein, CNBr1, T1, E1a, and E1b, while the C-terminus was proved as Gln in CNBr3 and E9. Cysteine is absent from the protein as judged by both amino acid analysis of the carboxymethylated protein,

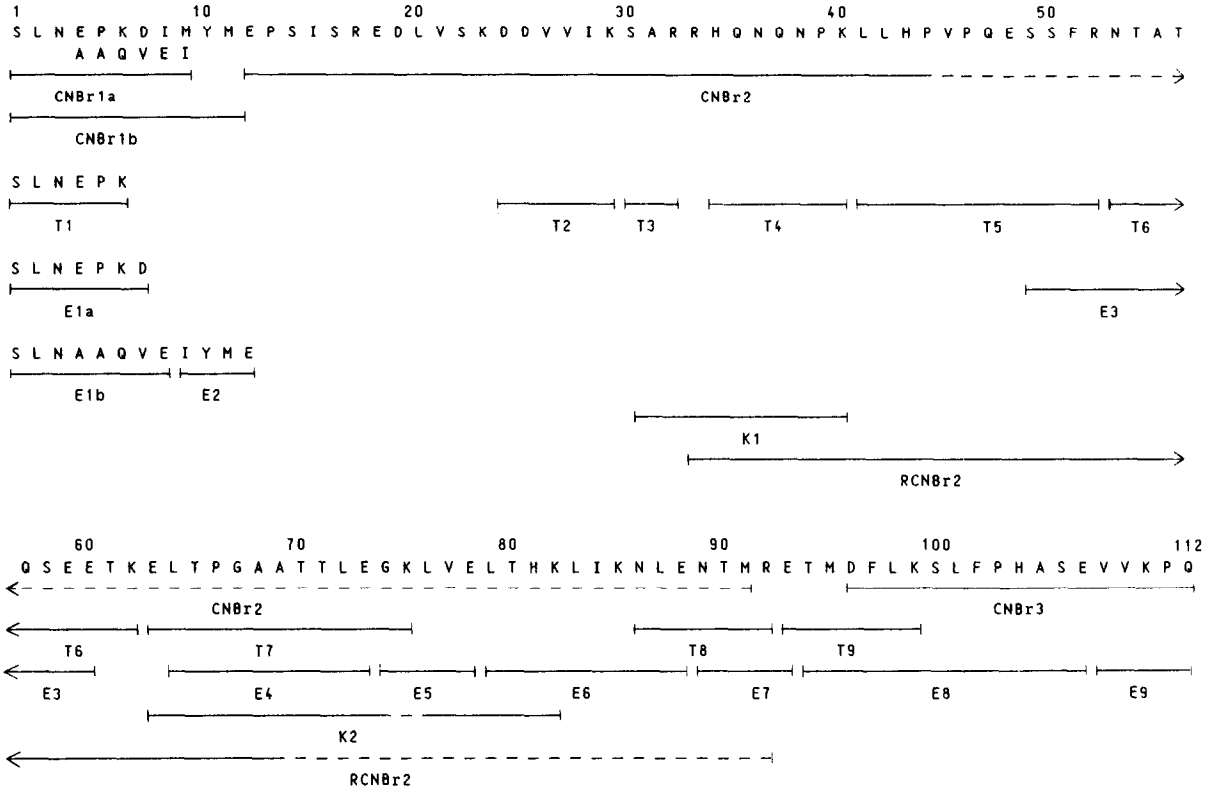

Fig.2. The structure deduced for the camel milk whey protein. The positions and extent of analysis of all peptides are indicated by the lines. Solid lines indicate regions analyzed by sequence degradations as given in section 2, dashed lines regions estimated by total compositions only. CNBr1–3 are cyanogen bromide fragments obtained by reverse-phase high-performance liquid chromatography (fig.3). T1–9 are the tryptic peptides, E1–9 the peptides obtained by digestion with Glu-specific protease, K1 and K2 the peptides obtained by digestion with the Lys-specific protease, and RCNBr2 the fragment obtained by cleavage of CNBr2 with clostripain at Arg. CNBr1a and 1b denote the two types of the N-terminal CNBr fragments (analyzed in mixture); their heterogeneity is supported by the same results with two E1 peptides (E1a and E1b, analyzed separately after purification). Direct degradation of the intact protein also revealed both structures. Additional heterogeneities are not excluded, in which case the structure determined may represent elements from either chain at single positions.

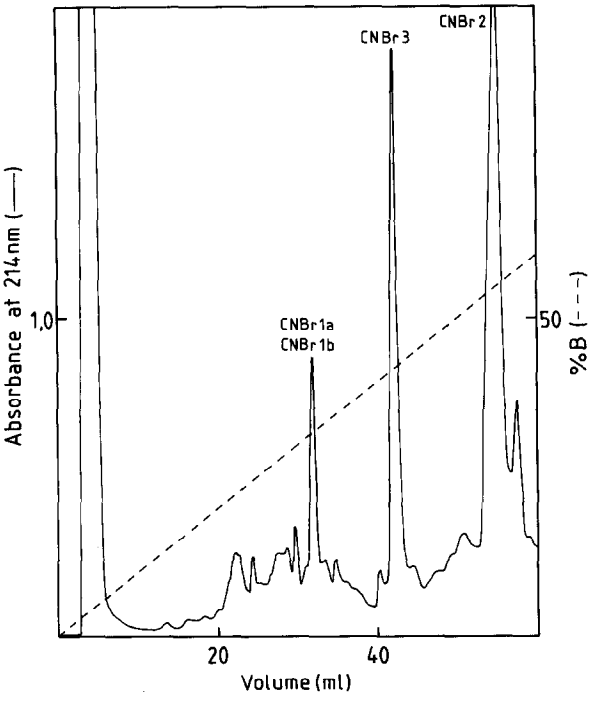

Fig.3. Purification of the CNBr fragments. Chromatographic conditions as in fig.1, and peptide designation as in fig.2.

and sequence analysis of the purified fragments.

A small fragment (peak 1, fig.2 [6]) was also isolated and found to represent the N-terminal part of the whole camel protein, showing the presence in milk of fragments of the intact protein. On sequence analysis of the native fragment, the parent protein, and the N-terminal CNBr fragment (CNBr1), the presence of two residues was observed during steps 4–9. Thus, an apparent heterogeneity of the camel milk protein was deduced.

4. DISCUSSION

4.1. *Primary structure and heterogeneity*

The structure deduced consists of 112 residues and does not contain cysteine. In the N-terminal segment, two structures were identified (fig.2) in equal amount (as judged by recoveries of PTH amino acids during degradation of the intact peptide). Although quite extensive differences between the two chains were found at six positions in the N-terminal regions, and further differences therefore should not be excluded, heterogeneities elsewhere in the molecule were not positively identified. However, if such heterogeneities are present, the structure deduced need not entirely represent one form but could, at single positions, contain elements of either form.

4.2. *Processing of the parent protein*

The identification of a small fragment corresponding to the N-terminal part of the parent protein shows that this protein has a cleavage pattern with the occurrence of fragments in milk, in a manner similar to that already known for β-caseins, in which case morphinomimetic [12,13] and other fragments [14] have been found. Both the native fragment and the intact protein revealed similar N-terminal heterogeneities, suggesting the presence originally of different types of protein chain.

4.3. *Nature of the protein*

No obvious structural similarities were noted between the novel milk protein and other characterized milk proteins. Although the present protein is apparently common in the camel milk whey, it is possible that it, like some other milk proteins [4,6] has a limited distribution, being a major component in milk of only some species. Some superficial similarities in properties (but not structures) with β-lactoglobulins may be noted. β-Lactoglobulin is common in milk of some but not all [3] species, and occurs in forms with characterized variants [19,20]. The present results define another unique type of protein in camel milk, and suggest the presence of various types of this protein.

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