Regulatory Peptides, 15 (1986) 55–62
Elsevier

RPT 00490

Characterization of a camel milk protein rich in proline identifies a new β-casein fragment

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(Received 13 February 1986; revised manuscript received 20 April 1986; accepted for publication 28 April 1986)

Summary

A camel milk whey protein has been isolated by reverse-phase high performance liquid chromatography. The protein is, like caseins, rich in proline (25% of the whole protein). The N-terminal amino acid sequence shows that the protein is homologous with a C-terminal region of β-caseins analyzed from other species. The protein is concluded to be a fragment of β-casein, derived from a non-tryptic type of cleavage of the parent molecule, and increasing the multiplicity of known casein products.

amino acid sequence; camel protein; milk whey; casein; opioid peptides

Introduction

Several fragments corresponding to N- and C-terminal parts of β-casein have been characterized from bovine milk [1,2]. These fragments include residues 29–209 known as γ_1-casein, residues 106–209 known as γ_2-casein, and residues 108–209 known as γ_3-casein [3,4]. Proteose-peptone fractions of milk also contain fragments of β-casein, which include the N-terminal residues 1–28 known as component 8 fast [5], residues 29–105 and 29–107, known as component 8 slow [6], and residues 1–105 and 1–107 known as component 5 [3]. It has been suggested that these fragments are obtained by limited proteolysis of β-casein [7]. Similar fragments have been obtained by plas-

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min mediated proteolysis of casein in bovine milk [8]. Other studies on proteose-peptone fractions from bovine milk have resulted in the characterization of opioid peptides [9–11] which have also been synthesized by chemical methods [11]. Recently a μ-opiate receptor-specific peptide, morphiceptin, was isolated from enzymic digest of caseins [12]. The presence of opioid sequences has also been proposed in characterized human milk β-casein [13].

In attempts to characterize camel milk proteins [14–16], we have purified a small protein rich in proline. On structural analysis it was found to be homologous to the C-terminal part of characterized β-caseins [4,13,17] from other species. Complete structure elucidation suggests that it is a fragment of camel β-casein, from which a new cleavage pattern in β-caseins can be deduced, and additional species variants of casein can be judged.

Materials and Methods

Whole milk from *Camelus dromedarius* was treated as described [15] and submitted to chromatography on Sephadex G-100 (50 × 2.5 cm) in 5% acetic acid. This gives four fractions which have been reported previously [14,15]. The fraction with α-lactalbumin as the major component was further separated by reverse-phase HPLC on a μBondapak C18 column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The proline-rich peptide now studied eluted as a minor component directly after the α-lactalbumin fraction, as previously described (peak 4 in the HPLC pattern shown in Fig. 2 of Ref. 14). Contaminating α-lactalbumin was removed by re-chromatography using the same HPLC system.

One sample (40 nmol) was cleaved with CNBr (200 mg) in 70% formic acid at room temperature for 24 h, while other samples (30 nmol) were used for enzymatic digestions with trypsin and a Glu-specific protease (*Staphylococcus aureus*, strain V8; Miles Laboratories, Slough, U.K.) in 0.2 M ammonium bicarbonate, pH 8.0, 37°C, 4 h, at enzyme to substrate ratios of 1:100. Peptide purifications were carried out by reverse phase HPLC under the same conditions as for the intact peptide.

The intact peptide, the CNBr fragments, and the enzymatic peptides were submitted to sequence analysis by the manual DABITC method [18,19]. Large peptides were analyzed by sequencer degradations in a Beckman 890C liquid phase instrument with glycine-precycled polybrene [20] or in an Applied Biosystems 470A gas-phase sequencer. PTH amino acids were identified by reverse phase HPLC [21]. Amino acid compositions were determined with a Beckman 121M analyzer after hydrolysis in evacuated tubes at 110°C for 24 h with 6 M HCl/0.5% phenol.

Results

The new protein was purified from camel milk by fractionation on Sephadex G-100, followed by reverse-phase HPLC purification [14] as described in Materials and Methods.

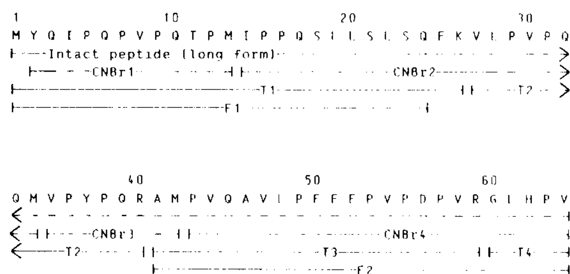

Fig. 1. The primary structure of the camel milk protein corresponding to a new type of casein fragment. The structure given is that of the major form ('long form') of the protein. In addition, a minor component was detected and corresponds to a 1-residue shorter peptide starting at position 2, as mentioned in the text. All peptides analyzed to determine the structure are shown. Abbreviations for amino acids are given by the one-letter code. Solid lines indicate residues proven by sequence analysis in a peptide, dashed lines show analysis by total compositions only. CNBr1–CNBr4 are CNBr fragments, T1–T4 tryptic peptides, and E1–E2 peptides from cleavages with staphylococcal Glu-specific protease. (E2 originates from a cleavage site atypical for the enzyme used, E1 presumably from a site produced by deamidation of residue 24.)

A 64-residue amino acid sequence was established, as shown in Fig. 1, by analysis of the intact protein, and the peptides obtained by treatments with CNBr, trypsin and Glu-specific protease. Some lactalbumin contamination of the preparation did not interfere with the analysis, since the lactalbumin content was low (legend, Table

TABLE I

Amino acid composition of the intact camel milk β-casein fragment

Peptide	
Asx	1.0 (1)
Thr	1.4 (1)
Ser	3.0 (3)
Glx	10.5(11)
Pro	16.0(16)
Gly	1.4 (1)
Ala	2.1 (2)
Val	9.5 (9)
Met	4.0 (4)
Ile	2.1 (2)
Leu	6.4 (6)
Tyr	1.8 (2)
Phe	2.0 (2)
Trp	1.0 (1)
Lys	1.0 (1)
His	2.0 (2)
Sum	64

Values given are those obtained after correction for contamination of α-lactalbumin (12%) in the preparation.

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                           10                  20                  30
Camel     M Y Q I P Q P V P Q T P M I P P Q S L L S L S Q F K V L P V P Q Q M V P Y P Q
Human   S   Q   V P     I   Q   L A L       P L W   V P E P         I   Q E V L P Y   V
Bovine  W   H   P H     L   P   V M F       S V L   L S Q S         V   E K A V P Y   Q
Ovine   W   H   P P     L   P   V M F       S V L   L S Q P         V   Q K A V - -   Q

       40                  50                  60
        R A M P V Q A V L P F E E P V P D P V R G L H P V
          A V   V     L   L N Q E L L L N   P H Q I Y   V P E P S T T Z A B H P I S V
          D M   I     F   L Y Q Q P V L G   V R G P F   I I V
          D M   I     F   L Y Q E P V L G   V R G P F   I L V
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Fig. 2. Comparison of the camel milk β-casein fragment now analyzed (top line with residues numbered as in Fig. 1) and corresponding parts of bovine, ovine and human β-caseins [4,13,17] (starting at position Met-144 of the bovine protein). The first W in the bovine line is Trp-143. For the camel protein, all residues are given in the top line; for the other proteins, only those residues that differ are shown in remaining lines. Thus, empty spaces indicate identities (except for the C-terminal parts where all structures are included up to their ends). Gaps are shown by dashes.

I) and since that structure was already known [15]. The amino acid composition of the whole protein now determined is given in Table I.

The structure obtained is homologous to positions 144–207 of bovine β-casein. Hence, since large parts of the structure are not unique, independent proof for all residue assignments are not reported. However, complete data are available, as summarized in Fig. 1, and fully agree with the structure deduced.

N-terminal heterogeneity

During sequence degradation of the intact peptide and its N-terminal tryptic peptide, a heterogeneity was noticed. Thus, a minor fraction corresponded to an amino acid sequence shifted by one residue, starting at position 2 of the whole protein. The ratio of the two amino acid sequences, as calculated from the yields of PTH-amino acids, was 3:1 for the whole protein versus the 1-residue shorter molecule.

Comparison with C-terminal fragments of bovine, ovine and human β-casein

The new camel milk protein is compared with C-terminal parts of β-caseins from bovine, ovine and human milk in Fig. 2. In total, 24 residues are conserved of those at the 64 positions compared.

The camel milk β-casein fragment is apparently two residues shorter at the C-terminus than bovine and ovine β-casein. However, both these β-caseins are shorter than the human form (Fig. 2), suggesting differences in C-terminal processing or ends of translation among caseins from different species.

Comparison with characterized opioid peptides

Characterized casein fragments with known opioid activity contain proline residues in alternating positions. Four such structures are also found in the camel casein fragment now determined, as shown in Fig. 3. The similarities affect only the proline

Peptide	Structure	Positions
β-casein fragment (camel)	I P Q P V P Q	4-10
	L P V P Q Q M	28-34
	V P Y P Q R A	35-41
	E P V P D P V	52-58
β-casomorphin-7 (bovine)	Y P F P G P I	60-66
β-casomorphin-5 (bovine)	Y P F P G	60-64
Morphiceptin (bovine)	Y P F P-NH_2	60-63

Fig. 3. Comparison of segments containing alternating proline residues of the camel β-casein fragment with structures of β-casomorphins [10] and related peptides [12]. Positional numbers refer to the present structure for the camel protein (top four entries) and to β-casein for the bovine peptides (bottom three entries). The C-terminal proline residue in morphiceptin is amidated [12]. This is consistent with the presence of Gly in the next position of β-casomorphins, since the amido group is derived from Gly [22]. Proline residues are shaded.

residues, emphasizing their conservation and suggesting important functions to these regions. However, no biological activity has so far been reported for fragments of camel milk proteins.

Discussion

Structural analysis

The structure consists of 64 residues and was determined by sequence analysis of the intact protein fragment and its sub-peptides. The sequence is consistent with the total composition (Table I) and shows residue exchanges at a total of 40 positions between camel and any of the previously characterized caseins (Fig. 2). The proline content is high (25%, Table I). Of these proline residues, most (11 of 16) are strictly conserved in all species, showing the importance of the proline distribution, which is typical of caseins.

New type of fragmentation of β-casein

The results show that the camel milk protein is homologous with a C-terminal fragment of β-casein. The peptide is concluded to originate from a major cleavage after Trp or Ser, as deduced from preceding residues in the homologous β-caseins (Fig. 2). The second fragment now detected, present in minor amounts, and starting at position 2 of the major fragment, is similarly cleaved after Met-1. Thus, it may be concluded that the processing enzyme(s) show chymotryptic-like activity. This pattern in the casein fragmentation is in contrast to that in many other peptide systems, where fragmentations are frequently of a tryptic-like specificity, as in the family of previously known casein-fragments [3–8]. Consequently, the new casein fragment defines the presence of a second type of cleavage fragments among the casein products.

Opioid peptides

The structure determined contains no less than four regions characterized by alternating proline residues (Fig. 3), which is one of the properties typical of peptides with opioid activity that have previously been characterized from proteose-peptone fractions of bovine milk [9–12]. The opioid peptides represent positions 60–63, 60–64 and 60–66 in the bovine β-casein, are resistant to pronase, and occur as homologous sequences in other species. The smallest biologically active structure is that of morphiceptin, which is a tetrapeptide [12].

It is possible that the large β-casein fragment now characterized in camel milk, as well as several of the previously isolated smaller casein fragments in other species, have some functional importance in vivo. If so, releases or regulations could perhaps explain the reports of many different casein fragments in milk, including possibly the present characterization of a second set of fragments generated by a chymotryptic-like type of activity.

Acknowledgement

This work was supported by grants from the Swedish Medical Research Council (project 03X-3532), the Swedish Cancer Society (project 1806), the Magn. Bergvall Foundation, and the Knut and Alice Wallenberg Foundation. Fellowships from the International Seminar in Chemistry, University of Uppsala, Sweden, to O.U.B. made this study possible.

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