Casocidin-I: a casein-α_{s2} derived peptide exhibits antibacterial activity

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Abstract Here we report the isolation and characterization of an antibacterial peptide from bovine milk inhibiting the growth of *Escherichia coli*, and *Staphylococcus carnosus*. The primary structure of the peptide was revealed as a 39-amino-acid-containing fragment of bovine α_{s2}-casein (position 165–203) by means of Edman amino acid sequencing and mass spectrometry. Since human milk does not contain any casein-α_{s2}, these findings could explain the different influence of human and bovine milk on the gastrointestinal flora of the suckling.

Key words: Antibacterial; Peptide; Milk; Casein; Intestinal flora

1. Introduction

The occurrence of certain bacterial species in milk indicates the existence of specific factors influencing bacterial growth. Another finding is that the intestinal flora of the newborn mainly depends on the source of the ingested milk [1]. Breast-fed sucklings, for example, almost exclusively exhibit *Bifidobacterium bifidum* in their intestine [2]. Milk proteins which are supposed to be responsible for this modulation are for example lysozyme [3,4], lactoperoxidase [5–7], lactoferrin [7–9] and antibodies [10]. However, in vivo the ingestion of lactoferrin has only a marginal influence on the intestinal microflora [11]. Moreover, it was shown that different effects on the microbial growth can be obtained by applying certain dietary formulations of milk proteins [11].

In the last few years, the research on antimicrobial peptides like defensins has come into focus [12]. These peptides were first identified in insects [13] and are also present in lower vertebrates and in mammals. Antimicrobial peptides are mainly stored in cytoplasmic granules of specialized leukocytes [14] but the presence of these factors could also be demonstrated in intestinal Paneth cells of mice (cryptdins) and man (defensins), indicating a possible role in the regulation of the local bacterial flora [15–19]. Another aspect is that several antimicrobial peptides present in ruminants are not found in other mammalian species, possibly correlating with the different anatomy and physiology of their gastrointestinal system [20,21].

The aim of this study was to investigate whether peptides capable of modulating the composition of the intestinal flora are present in bovine milk. In a whey fraction we were able to detect a heat and acid-stable antibacterial property against *E. coli*. Further purification and characterization revealed a 39-amino-acid-containing fragment derived from casein-α_{s2} to which we refer as casocidin-I. Many bioactive peptides have earlier been identified as digestion products of caseins. Some of them behave like opioids, immunomodulators or enzyme inhibitors. Others have antithrombotic activities or act as mineral carriers [22–24].

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2. Experimental

2.1. Treatment of milk

Grade A bovine milk (Heirler, Darmstadt, Germany; 1.5 litres of controlled quality) was purchased from a local health-food shop. Glacial acetic acid was added to a final concentration of 10% (v/v) and the milk was subsequently boiled for 5 min. It was then cooled to 50° C and 1 g/l $CaCl_2 \cdot 2H_2O$ was added. To block serine proteases we used 50 μM phenylmethylsulfonate. After centrifugation (15 min, 3500 × *g* at 4°C) the resulting supernatant was collected, diluted with 4 volumes of water and incubated with 25 ml of a strong cation exchange resin (Parcomer; 20 μm, Biotek, Heidelberg, Germany). The resin was washed with 250 ml of 5 M urea in 5 mM phosphate buffer (pH 3.0) and with 250 ml water. To elute bound peptides we applied 100 ml of 1 M NaCl in 5 mM phosphate buffer (pH 3.0) at room temperature. The eluate was centrifuged (5 min, 1000 × *g*, RT) and residual resin particles were removed by means of filtration through a 0.2 μm disposable syringe filter (Sartorius, Göttingen, Germany).

2.2. Antibacterial assay

Column fractions were tested against *Escherichia coli* XL1 blue (Stratagene, Heidelberg, Germany), *Escherichia coli* BL21 (DE3) and *Staphylococcus carnosus* TM300 (a gift from F. Goetz, Technical University of Munich, Germany) by a sensitive radial diffusion technique as described [25]. We used agarose without EEO (Serva, Heidelberg, Germany) either in 25 mM phosphate (pH 7.2) or Tris-HCl (pH 7.4).

2.3. HPLC purification

The HPLC purification steps were performed on Parcosil-C_{18} (1 × 12.5 cm, 100 Å, 5 μm) and Parcosil-C_4 (0.4 × 12.5 cm, 300 Å, 5 μm) reversed-phase columns (Biotek, Heidelberg, Germany). A HPLC system (Kontron, München, Germany) equipped with two model 420 pumps, a model 432 fixed-wavelength detector (214 nm or 280 nm) and a model 450 data system was used. After equilibration with 0.1% TFA, peptides were eluted by linearly increasing the amount of solvent B (acetonitrile with 0.1% TFA) as follows: gradient 1 = 0–50 min, 0–80% B; gradient 2 = 0–5 min, 0–15% B; 5–65 min, 15–80% B; gradient 3 = 0–5 min, 0–15% B; 5–65 min, 15–80% B.

2.4. Proteolytic cleavage

Proteolytic cleavage was performed with trypsin, pronase or endoproteinase Glu-C in 25 mM Tris-HCl, pH 7.0, at a protease-to-peptide ratio of 1:100 (w/w). The reactions were stopped by heating at 95°C for 5 min. Samples without enzyme were used as controls.

2.5. Peptide analysis

Amino acid sequence determinations were carried out automatically on an Applied Biosystems 473 A gas phase sequencer (Applied Biosystems Div. of Perkin Elmer, Weiterstadt, Germany).

2.6. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was performed with a 50 cm uncoated capillary of fused silica on the CZE system model P/ACE 2100 (Beckmann, München, Germany). The running buffer used was 100

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mM NaH_2PO_4, pH 2.5, containing 0.02% hydroxypropylmethylcellulose. After injection of 60 nl sample, the separation was carried out with a constant current of 120 μA.

2.7. Mass spectrometric analysis (MS)

Mass spectrometric analysis (MS) was performed on a triple-stage quadrupole electrospray mass spectrometer Sciex API III (Perkin Elmer, Ueberlingen, Germany) equipped with an articulated ion-spray source operating at atmospheric pressure. The lyophilized samples were dissolved in 50% acetonitrile/0.2% acetic acid prior to injection. Mass spectra were recorded in positive ion mode. For the HPLC-MS analysis, a reversed phase C_{18} column (YMC, Schermbeck, Germany, 1 mm × 250 mm, 120 Å, 3 μm) and the mass spectrometer were coupled. Direct coupling of HPLC with the mass spectrometer was performed using a model 140B double syringe pump, and a model 740B UV-detector equipped with the capillary flow cell (Applied Biosystems Div. of Perkin Elmer, Weiterstadt, Germany). The flow rate was 20 μl/min using a linear gradient starting with 0.06% TFA increasing the eluent B (80% acetonitrile with 0.05% TFA) by 1% per minute. The column outlet was directly connected with the ion spray using a 50 μm fused silica capillary without post column splitting.

2.8. Chemical synthesis

Chemical synthesis of peptides was carried out as described elsewhere [26,27]. The purification was performed on a Vydac RP-C_{18} column (MZ-Analysentechnik, Mainz, Germany; 10 μm, 300 Å, 2 × 25 cm) with 0.06% TFA/acetonitrile/water at a flow rate of 10 ml/min. The purity was controlled by means of HPLC, CZE and mass spectrometry.

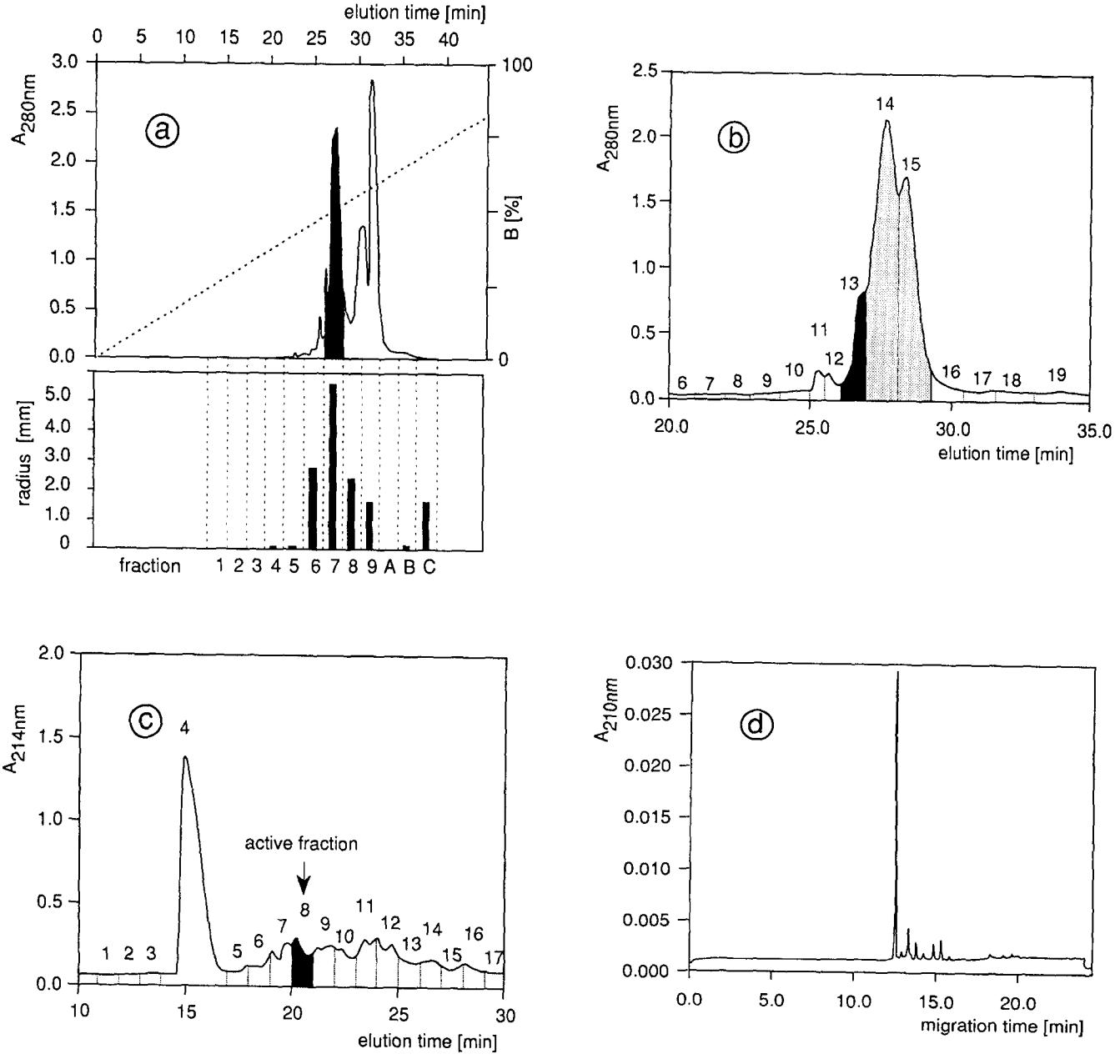

Fig. 1. Purification of casocidin-I. (a, top) The eluate of the cation exchanger equivalent to 350 ml whey was chromatographed on a Parcosil C-18 column using gradient 1. (a, bottom) The growth inhibitory activity of the eluate was monitored with *E. coli* XL-1 blue as described in methods. The radius of the growth inhibition zone sorrounding the wells was measured. (b) The most active fraction 7 was rechromatographed applying gradient 2 on the same column. Antibacterial fractions are shaded. Fraction 13 is further processed. (c) RP-C4 chromatography of fraction 13 using gradient 3. The active fraction 8 is seen in the hatched zone. (d) CZE analysis of fraction 8 containing casocidin-I.

39
KTKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQK

Fig. 2. Amino acid sequence of casocidin-I. Casein-α_{s2}(165–203) in the one letter code. Basic residues are typed in boldface.

3. Results and discussion

3.1. Purification of casocidin-I

In order to denature and to clear away most of the high molecular weight proteins, we initially treated the milk by boiling and mild acidifying. Additionally, calcium chloride was added to precipitate calcium-dependent phosphoproteins [28]. Fat and coagulated proteins were removed by means of centrifugation, the resulting volume of the supernatant was about 66%. To concentrate basic peptides, a cation exchange step was performed as described above. For further purification of the antibacterial peptides, we combined different reversed-phase HPLC separation procedures. For the first separation step a Parcosil C_{18} RP column was used and an amount of 350 ml whey (Fig. 1a) was applied. The bactericidal activity of an aliquot equivalent to 8.8 ml whey was monitored, and the maximum activity could be detected in fraction 7 (Fig. 1a). Lower levels could also be detected in adjacent fractions and in fraction C. Prior to purification procedures we performed some controls: lysozyme activity was monitored with a *Micrococcus lysedeicticus* cell wall suspension and the presence of β-lactam antibiotics was excluded using bacteria carrying the plasmid pUC-18. At least we were able to eliminate the antibacterial activity with trypsin or pronase indicating that it was coupled to a polypeptide. Thus, further purifications were conducted. The second step of purification was performed with the same RP-C_{18} column using a less steep gradient (Fig. 1b). Here we tested the eluate for the presence of antibacterial activity which eluted over a broad range in fractions 13–15. Further HPLC purification steps using fractions 14 and 15 could resolve several peaks but resulted in the loss of activity. In the third step of purification, the antibacterial activity of fraction 13 could be recovered by RP-C_4 HPLC (Fig. 1c). This appeared in fraction 8, whereas fraction 4, representing a dominant peptide-containing peak, exhibited no inhibitory effect.

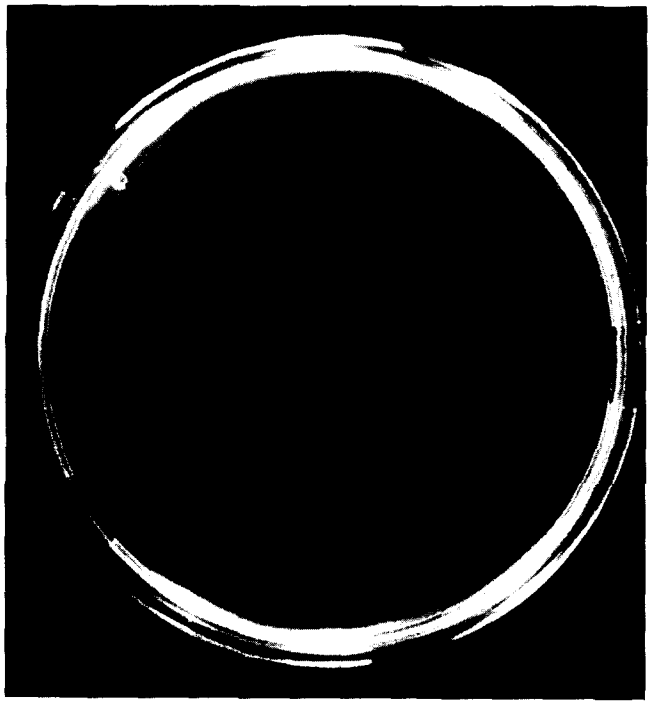

Fig. 3. Growth inhibition test with *Staphylococcus carnosus* TM300. Each well was loaded with 10 μl sample containing various amounts of casocidin-I. The arrow indicates the direction of the increasing peptide dosage (range 0 ng, 100 ng, 200 ng, ... 1400 ng peptide). The diameter of each well is 3 mm. The dark areas correspond to the dose dependent zones of growth inhibition after application of synthetic casocidin-I.

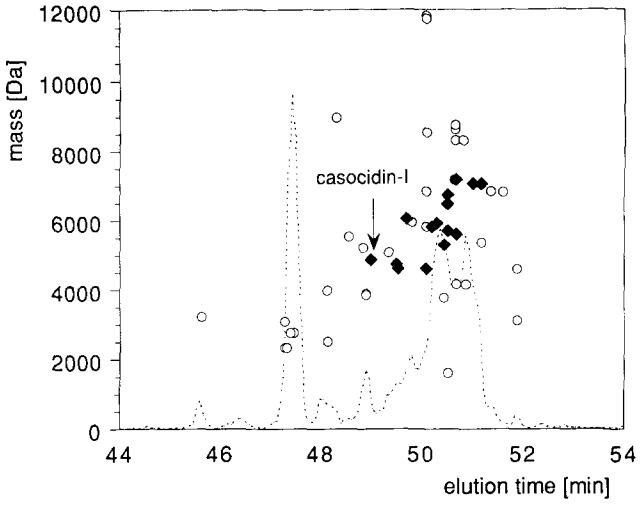

Fig. 4. LC-MS analysis of the active fraction 7 from Fig. 1a. The dotted line shows the reconstructed ion current profile. Open circles indicate position and mass of several molecules. Solid diamonds represent casein-α_{s2} masses starting with residue 165 or 166.

3.2. Characterization of casocidin-I

CZE analysis revealed a dominant compound with high purity (Fig. 1d) sufficient for amino acid sequence and mass spectrometric analysis. The peptide was identified as a 39-amino-acid-containing casein-α_{s2} fragment (position 165–203) of 4870 Da which we refer to as casocidin-I (Fig. 2). Both terminal amino acids of the peptide represent potential tryptic cleavage sites. Moreover, the N-terminal lysine is located in a dibasic lysine motif appearing in the precursor molecule. The calculated pI of this peptide is 8.9 and a hydrophobicity-plot shows a regular alternation of hydrophobic and hydrophilic residues. This gives rise to the hypothesis that the mechanism of action resembles that of amphiphatic defensins permeabilizing bacterial membranes. Casein-α_{s2} is not present in human milk and so we propose that casocidin-I or related peptides of bovine milk influence the human intestinal flora, particularly of the suckling.

3.3. Antibacterial activity of synthetic casocidin-I

In order to demonstrate that the activity was due to casocidin-I and not to accompanying impurities, we chemically synthesized casein-α_{s2}(165–203). Subsequently performed bioassays showed that the synthetic peptide also exhibits an antibacterial effect. Using *E. coli* XL1 blue, *E. coli* BL21, and

Staphylococcus carnosus TM300 casocidin-I induces a dose dependent zone of growth inhibition in the used assay (Fig. 3). *S. carnosus* appeared to be more sensitive than *E. coli*. Here, only 1/10 of casocidin-I is necessary to generate comparable growth inhibitory effects. The antibacterial properties vanished after degradation with trypsine but not after treatment with endoproteinase Glu-C. So the glutamate residues near the N-terminus did not seem to be essential for the growth inhibition.

3.4. HPLC-MS analysis of active fractions

To estimate the number of peptides, and the presence of casocidin-related peptides in the starting fraction 7 we applied a high resolution C_{18} reversed phase HPLC separation step coupled with the quadrupole ion spray mass spectrometer as a detector (HPLC-MS analysis). By use of this method we detected several distinct masses appearing in the elution profile. To prove our assumption that peptides homologous to casocidin-I, possibly sharing the same antibacterial property, were present in fraction 7, we computed the compound masses of hypothetical casein-α_{s2}-derived fragments beginning with residue 165 or 166. We were able to identify a large number of the corresponding masses (Fig. 4). For example, the mass for the complete C-terminal portion of casein-α_{s2} was also detectable. We assume that these fragments together combine their antibacterial activity. The difficulty in recovering the activities of certain subfractions might have been due to the large number of only slightly differing molecules, each present in a lower amount than the detection limit of the bacterial growth inhibition assay.

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