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Purification of novel peptide antibiotics from human milk

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Abstract

A strategy was established for the identification of novel antimicrobial peptides from human milk. For the generation of bioactive peptides human milk was acidified and proteolyzed with pepsin simulating the digest in infants stomachs. Separation of proteins and resulting fragments was performed by means of reversed-phase chromatography detecting the antimicrobial activity of each fraction using a sensitive radial diffusion assay. In order to avoid the purification of the known abundant antimicrobial milk protein lysozyme, it was identified in HPLC fractions by its enzymatic activity and by matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS). On condition that lysozyme was not detectable and antibacterial activity of HPLC fractions was caused by a peptide, which was confirmed by proteolytic cleavage leading to a loss of activity, further purification was performed by consecutive chromatographic steps guided by the antibacterial assay. Using this strategy, an as yet unknown casein fragment exhibiting antimicrobial activity was purified in addition to antimicrobial lactoferrin fragments. The new antimicrobial peptide resembles a proteolytic fragment of human casein-κ (residues 63–117) and inhibits the growth of Gram-positive, Gram-negative bacteria, and yeasts. Our results confirm that antimicrobially-active peptides are liberated from human milk proteins during proteolytic hydrolysis and may play an important role in the host defense system of the newborn.

Keywords: Peptide analysis

1. Introduction

The antibacterial properties of human and bovine milk have been known for a long time. The use of milk to prevent bacterial infections has been described in ancient and medieval publications [1]. The incidence of disease, especially diarrhea, is significantly lower in breast-fed infants than in formula-fed infants and a variety of protective factors in human milk are claimed to be responsible for this. Known bacterial growth inhibiting compounds in milk are lysozyme (for review, see [2]), lactoperoxidase [3] and lactoferrin (for review see [4]).

The digestion of milk proteins represents an important mechanism to obtain peptides which exhibit significant physiological roles in addition to their nutritional importance. Numerous opiate-like, antihypertensive, immunomodulating or antithrombotic protein fragments have been described (reviewed in [5] and [6]). Small proteolytic fragments of both bovine and human lactoferrin have been shown to exert antimicrobial activity in addition to

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the bacteriostatic activity of whole lactoferrin, which was thought to act by depriving microorganisms of iron [7]. In the late 1960s, Lahov and Regelson observed that hydrolysis of bovine milk casein by heating and chymosin treatment leads to the formation of polycationic low molecular mass peptides with antimicrobial properties, the so-called casecidines [8]. Known antimicrobial fragments from bovine casein are isracedin (casein-α_{s1} (16–38)) [9], casocidin-I (casein-α_{s2} (165–203) [10] and the casein-κ-derived glycomacropeptide [11,12]. Recently, Recio and Visser identified two distinct antibacterial domains (casein-α_{s2} (183–207) and casein-α_{s2} (164–179)) within the sequence of bovine casein-α_{s2} [13], corresponding to the sequence of casocidin-I. In contrast to casocidin-I, which was purified from the whey fraction of bovine milk, the peptides casein-α_{s2} (183–207) and casein-α_{s2} (164–179) were isolated from a peptic hydrolysate of bovine casein-α_{s2}. From human milk, no antimicrobial casein-derived peptide has yet been identified. Since human milk does not contain casein-α_{s2}, yielding polycationic antimicrobial peptides, it was interesting to clarify if there are equivalent peptides in human milk.

The aim of this study was to investigate which peptides, capable of inhibiting the growth of microorganisms, are liberated from human milk protein during enzymatic hydrolysis. Based on the observation that human milk suppresses the growth of special bacteria in the intestine of breast-fed infants, human milk was partially hydrolyzed with pepsin, like in infants' stomach. Components of pepsin-treated human milk were separated using RP-HPLC and the resulting fractions were analyzed for antimicrobial activity. The occurrence of the antibacterial protein lysozyme in HPLC fractions was checked by detecting its lytic activity on bacterial cell walls and by MALDI–MS. In order to identify novel antibacterial peptides from human milk, further purification was conducted by consecutive HPLC steps, if antibacterial activity of HPLC fractions was eliminated by proteolytic cleavage with the proteinase subtilisin and if no lysozyme was detectable.

Using this strategy, we were able to detect several antimicrobial peptides in pepsin-treated human milk and purified a novel peptide antibiotic, which was identified as a proteolytic fragment of casein-κ.

2. Experimental

2.1. Hydrolysis of human milk protein using pepsin

Human milk (collected 1–2 months after birth) was obtained from healthy voluntary donors after their informed consent. After acidification with HCl to pH 3.5, human milk was incubated with pepsin (20 mg/g protein) for 2 h at 37°C. The hydrolysis was stopped by boiling for 5 min. After centrifugation (20 min, 6000 *g* at 4°C), the fat was skimmed off and the supernatant was collected. Trifluoroacetic acid (TFA) was added to the supernatant to a final concentration of 0.1%. Floating particles were removed by filtration and repeated centrifugation (20 min, 2500 *g* at 4°C). The solution obtained was stored at −20°C.

2.2. Purification of antimicrobial peptides with hplc

For peptide purification, four subsequent HPLC steps were carried out, tracking the maximum antibacterial activity within the resulting fractions using *Staphylococcus carnosus* as a test strain. First HPLC step: RP-C_{18} column (Parcosil 1 cm×12.5 cm, 100 Å, 5 μm, Biotek, Östringen, Germany), solvent A: 0.1% TFA, solvent B: 0.1% TFA in acetonitrile, flow-rate: 2.0 ml/min, gradient: 0–60% B in 45 min. Second HPLC step: rechromatography of antimicrobial fractions with the same conditions as above using a shallower gradient (20–50% B in 45 min). Third HPLC step: the same RP-C_{18} column as in the first two purification steps was used changing solvent B to 0.1% TFA in methanol using a gradient of 40–70% B in 45 min. Fourth and final HPLC step: cation-exchange chromatography using a strong cation-exchange column (Parcosil Pepkat, 4×50 mm, 300 Å, 5 μm, Biotek), solvent A: 10 m*M* phosphate buffer (pH 4.5), solvent B: the same buffer with 1 *M* NaCl, flow-rate 0.75 ml/min, gradient: from 0 to 60% B within 60 min.

2.3. Peptide analysis

MALDI mass determination was carried out using a LaserTec RBT II (PerSeptive Biosystems,

Framingham, MA) as described previously [14]. The instrument is equipped with a 1.2 m flight tube and a 337-nm nitrogen laser. Positive ions are accelerated at 30 kV and up to 64 laser shots are automatically accumulated per sample position. α-Cyano-4-hydroxy-cinnamic acid (CHC, Sigma–Aldrich, Deisenhofen, Germany) was used as matrix. Accuracy of mass measurement was within 0.5%.

Purity of peptides was analyzed by capillary zone electrophoresis with a model P/ACE 2100 CZE system (Beckman, Munich, Germany).

Amino-acid sequencing was performed on a gas phase sequencer (model 473 A, Applied Biosystems, Weiterstadt, Germany) by Edman degradation using the fast cycle protocol as recommended by the manufacturer.

2.4. Peptide synthesis

Peptides were synthesized on a 433A peptide synthesizer (Perkin-Elmer/ABI) using standard Fmoc solid-phase chemistry [15]. Crude peptides were purified by reversed-phase HPLC (Vydac RP-C_{18}, 10 μm, 300 Å, 2×25 cm; MZ-Analysentechnik, Mainz, Germany) using 0.06% TFA in acetonitrile–water 4: 1 as solvent B. The purity and the identity of synthesized peptides was checked by analytical HPLC, mass spectrometric and sequence analysis.

2.5. Proteolytic cleavage

Proteolytic cleavage of bioactive fractions was performed with subtilisin (Boehringer Mannheim, Germany) in 100 m*M* Tris–HCl buffer pH 7.4 using an estimated protease to peptide ratio of 1:100 (w/w). After incubation for 2 h at 37°C the reaction was stopped by heating at 60°C for 2 min. Samples without enzyme were used as controls.

2.6. Antimicrobial assay

2.6.1. Microorganisms

Staphylococcus carnosus TM 300 was kindly provided by F. Goetz, University of Munich. *Escherichia coli* BL 21 was obtained from J. Alves, Hanover Medical School.

2.6.2. Radial diffusion assay

For detection of antimicrobial activity in HPLC fractions, a sensitive radial diffusion technique was used as described earlier [16]. Lyophilized HPLC fractions were dissolved in sterile water and subsequently tested against *Staphylococcus carnosus* TM 300 using a solid agarose medium. Agarose is used instead of agar since this medium does not inhibit peptide antibiotics [16]. The agarose layer consisted of 30 mg/100 ml Tryptic Soy Broth (Sigma, Germany) in 10 m*M* phosphate buffer (pH 7.2) with 0.02% Tween 20 and 0.8% GTG agarose (FMC BioProducts, Rockland, USA). The plates were incubated at 37°C for 16 h until growth of the microorganisms was visible. The diameters of the inhibition zone were recorded.

3. Results and discussion

3.1. Strategy

Human milk is a known source for substances influencing the growth of microorganisms. Breast-feeding exerts a regulatory effect on the composition of the intestinal microflora of sucklings by favoring the growth and maintenance of bifidobacteria and repressing the growth of other intestinal bacteria such as *Bacteroides* spp., clostridia and enterobacteria [17,18]. Therefore, we started to identify substances causing the antimicrobial effect of human milk.

Since human milk exhibits its bacterial growth modulatory effect following digestion in infants' gastrointestinal tract, we suppose that proteolytic hydrolysis of milk proteins results in the release of peptides capable of influencing bacterial growth. We expected the generation of peptide antibiotics comparable to the molecules found in bovine milk. To confirm this hypothesis, we compared biochemical and antimicrobial properties of acidified human milk before and after treatment with pepsin simulating the digestion of human milk in the sucklings' stomach. HPLC analysis of proteolyzed and non-proteolyzed milk samples revealed that treatment with the gastrointestinal protease pepsin resulted in high proteolytic degradation of human milk protein (Fig. 1A, B). This degradation was also visualized by

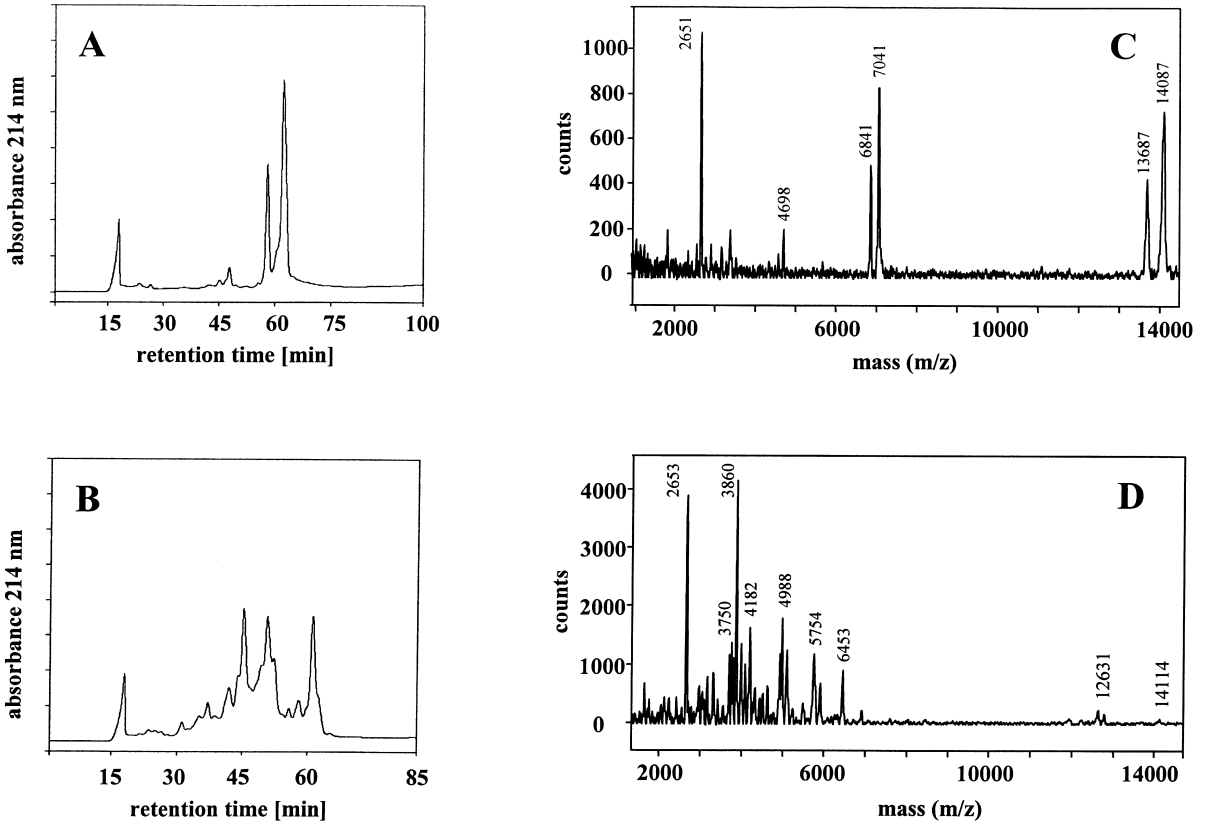


Fig. 1. Hydrolysis of acidified human milk with the gastrointestinal protease pepsin resulted in partial proteolytic degradation of milk proteins. Samples of non-proteolyzed (A) and of proteolyzed human milk (B) were analyzed by RP-HPLC (Source C_{15} column, 1 cm×12.5 cm, 300 Å, 5 μm, Pharmacia, Freiburg, Germany), revealing the generation of protein fragments. The degradation of milk protein was also visualized by MALDI–MS of non-proteolyzed (C) and proteolyzed human milk (D). Note the detected mass of 14 000 representing the known antimicrobial polypeptide lysozyme, which was mainly proteolyzed by pepsin as well.

MALDI–MS, which represents a fast and sensitive detection system for complex peptidic mixtures (Fig. 1C, D). In parallel to the generation of small proteolytic fragments, the antimicrobial activity was significantly increased after milk samples were subjected to pepsin digestion (data not shown). These findings confirm that peptides generated by hydrolysis of milk protein contribute to the antimicrobial activity of human milk.

To identify antimicrobially active protein fragments, hydrolyzed milk protein was first separated by means of reversed-phase HPLC (Fig. 2A). The antimicrobial activity of each fraction was monitored against *Staphylococcus carnosus* TM 300 as a sensitive test strain using a radial diffusion assay [16]. In order to avoid the purification of known antimicrobial milk components such as lysozyme, control experiments were performed prior to purification procedures. For detection of lysozyme in HPLC fractions, its lytic activity was monitored with a *Micrococcus lysedeicticus* cell wall suspension. Additional identification of lysozyme was performed using MALDI–MS. To confirm that the antimicrobial activity detected in HPLC fractions is caused by a polypeptide, proteolytic cleavage with subtilisin, a proteinase from *Bacillus subtilis*, was performed. If antibacterial activity of HPLC fractions was eliminated following proteolytic cleavage and no lysozyme was detectable, further purification was conducted by consecutive HPLC separation steps.

The purification procedure of antimicrobial peptides was guided by the radial diffusion assay

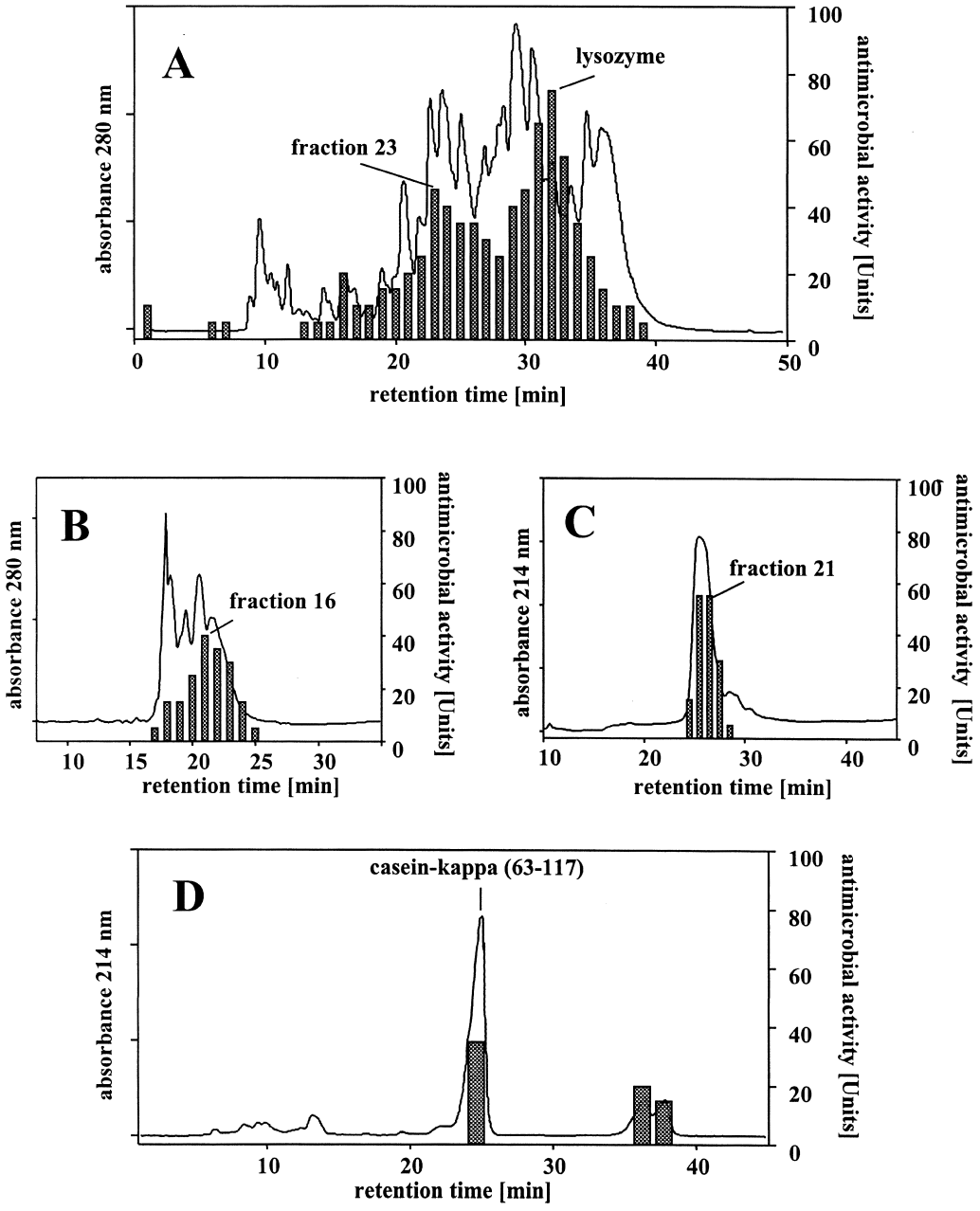


Fig. 2. Purification of an antimicrobially active peptide by consecutive chromatographic steps. Each purification step was guided by the radial diffusion assay for detection of antimicrobial activity. (A) First separation of 20 ml pepsin-treated human milk was performed by RP chromatography. The bars indicate the antimicrobial activity of an equivalent of 1 ml milk against *Staphylococcus carnosus* TM 300. One unit represents an activity corresponding to an inhibition zone of 0.1 mm radius. Lysozyme was identified in one of the two regions (fractions 31–33) revealing maximum antimicrobial activity. Fraction 23, corresponding to the maximum bacterial growth inhibitory activity, which was not caused by lysozyme, was selected for further purification. (B–C) Further purification steps using analytical RP chromatography. Antimicrobial activity was monitored by the radial diffusion assay applying an equivalent of 2 ml milk. (D) The last separation step using cation-exchange chromatography resulted in the purification of the peptide casein-κ (63–117) exhibiting antimicrobial activity.

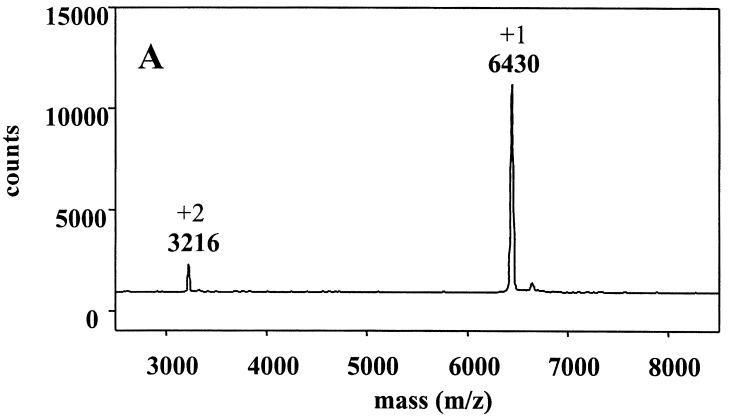
monitoring antibacterial activity of each HPLC fraction. For each separation step, fractions exhibiting the highest antibacterial activity were selected for further purification.

3.2. Purification of the antimicrobial peptide casein-κ (63–117)

For purification of antimicrobial human milk peptides, we combined different reversed-phase and cation-exchange HPLC steps. In the first separation step, 20 ml pepsin-treated human milk was applied to an analytical RP-C_{18} column. Polar compounds were removed by equilibrating the column with solvent A for 15 min and peptides were subsequently eluted by linearly increasing the amount of solvent B. The antimicrobial activity of each HPLC fraction corresponding to an equivalent of 1 ml milk was monitored (Fig. 2A). Bacterial growth inhibitory activity eluted over a broad range revealing maximum activity in two different regions of the chromatography. Antimicrobial activity detected in fractions 31–33 was caused by lysozyme as identified by its lytic activity and by MALDI–MS. In contrast, in fraction 23, which was a potent inhibitor of the growth of *Staphylococcus carnosus*, no lysozyme was detectable. The antimicrobial activity exhibited by fraction 23 was successfully eliminated after hydrolysis with subtilisin. Therefore, fraction 23 was selected for further purification.

The second purification step was performed with the same RP-C_{18} column using a less steep gradient (Fig. 2B). Antibacterial activity eluted over a broad range in fractions 12–20, showing its maximum in fraction 16. To achieve further separation in the subsequent purification step, selectivity was changed by using methanol with 0.1% TFA instead of acetonitrile with 0.1% TFA as solvent B (Fig. 2C). Corresponding to the main peak, maximum antimicrobial activity was detected in fractions 20 and 21. Since it is known that antimicrobial peptides have a cationic character, a strong cation-exchange column was used for final purification (Fig. 2D). The resulting HPLC fractions were desalted and subsequently tested for the presence of antimicrobial activity, which could be detected in one fraction corresponding to the main peak and in two adjacent fractions. Analysis of active fractions by CZE revealed compounds of high purity (data not shown), indicating that the developed purification strategy is well suited for the isolation of antimicrobial milk peptides. The peptides causing the antimicrobial activity detected in the side peaks were identified as fragments of lactoferrin very similar to the known bactericidal peptide lactoferricin H [19]. The MALDI mass spectrum of the main peak showed a mass of 6430 Da (Fig. 3A). The molecular mass was additionally determined by electrospray mass spectrometry, confirming the result obtained by MALDI–MS. N-terminal sequence analysis by conventional Edman degradation resulted in the sequence YQRRPAIAINNPYVPRTYYANPAVVRPHAQIP, which corresponds to a partial sequence of casein-κ. Considering the molecular mass of 6430 Da, the antimicrobially acting peptide can be matched to residues 63–117 within casein-κ. The peptide casein-κ (63–117) contains 55 amino acids and is non-glycosylated, which is confirmed by the calculated molecular mass of 6430.3 Da (Fig. 3B). These data demonstrate for the first time the purification of an antimicrobially active casein fragment from human milk.

The peptide bond 117–118 within casein-κ represents a known preferred cleavage site of pepsin, which splits casein-κ into two fragments, the hydrophilic glycomacropeptide (residues 118–182) and the hydrophobic para-κ-casein (residues 21–117). The human glycomacropeptide was already known to exhibit antimicrobial effects by its sugar composition. It inhibits *Helicobacter pylori* infection by interfering the adhesion of these bacteria to their target cells [11]. In addition, adhesion inhibitory properties of the glycomacropeptide towards *Haemophilus influenzae* and *Streptococcus pneumoniae* were reported [12]. However, the isolated casein-κ (63–117) does not contain glycosylation sites and therefore, the antimicrobial effect exhibited cannot be caused by carbohydrate content but requires a different explanation. Casein-κ (63–117) is a proline-rich cationic peptide with a calculated pI of 11.3. Therefore, it is likely that it belongs to the large group of polycationic antimicrobial peptides. Due to the high proline content of the peptide, an α-helical structure is improbable. Calculations of the



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1 55

YQRRPAIAINNPYVPRTYYANPAVVRPHAQIPQRQYLPNSHPPTVVRRPNLHPSF

Fig. 3. (A) MALDI–MS analysis of the purified antimicrobial peptide revealed a molecular mass of 6430 Da. (B) Sequence analysis by Edman degradation together with mass determination by MALDI–MS led to the identification of casein-κ (63–117). The amino acid sequence of casein-κ (63–117) is shown in the one letter code, the sequenced amino acids are presented in bold letters.

hydrophobic moment based on a planar confirmation revealed an amphipatic domain within the sequence of casein-κ (63–117). An amphiphilic structure is a common feature for antimicrobial peptides [20]. The amphipatic structure of casein-κ (63–117) gives rise to the hypothesis that its mechanism of action resembles that of known antimicrobial peptides such as magainins and defensins permeabilizing bacterial membranes [21].

3.3. Antibacterial activity of synthetic casein-κ (63–117)

In order to confirm that the detected antimicrobial activity was due to casein-κ (63–117) and not to accompanying impurities, the peptide was chemically synthesized. Subsequently performed bioassays showed that the synthetic peptide inhibits the growth of several Gram-positive and Gram-negative bacteria and yeasts (data not shown). Using the radial diffusion assay, casein-κ (63–117) induces a dose-dependent zone of growth inhibition against all bacteria tested (Fig. 4).

4. Conclusions

The proteolysis of acidified human milk with the gastrointestinal protease pepsin results in the generation of antimicrobially acting peptides. We established a combined strategy to identify the major components of human milk causing its antimicrobial activity. Creating experimental conditions similar to the conditions existing in infants' stomach, peptides capable of inhibiting the growth of microorganisms were liberated from human milk protein. Prior to purification procedures, the known antimicrobial milk protein lysozyme was detected by the use of a bioassay and mass spectrometric analysis. Unknown antimicrobial compounds were purified by means of HPLC using the radial diffusion assay for monitoring antimicrobial activity. Using the represented strategy, we were able to identify several low-molecular-mass peptides responsible for the antimicrobial activity of human milk protein. Besides known antimicrobial protein fragments such as peptides similar to lactoferricin H an as yet unknown antimicrobially active fragment of casein-κ was found and characterized. The results show that human milk provides many

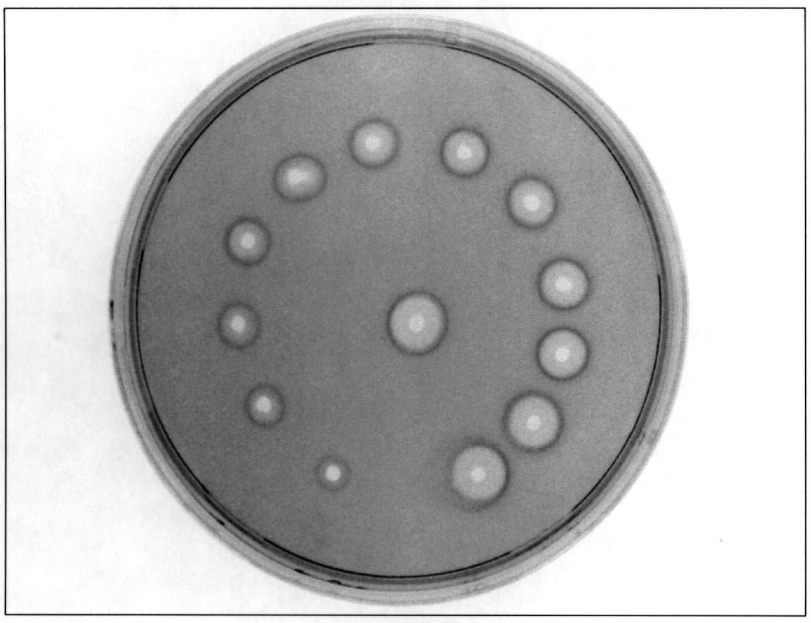

Fig. 4. Growth inhibition test with *Escherichia coli* BL 21. Each well was loaded with 10-μl sample containing various amounts of synthetic casein-κ (63–117). The applied peptide dosage (range 1 μg, 2 μg, ... 10 μg, 15 μg, 20 μg) increases clockwise. The clear areas correspond to the dose dependent zones of growth inhibition after incubation for 16–20 h at 37°C.

antimicrobially active polycationic peptides: lysozyme, lactoferricines and casein derived peptide antibiotics, which we termed casocidines.

Since the represented antimicrobial milk peptides are generated by hydrolysis of major milk proteins simulating the digestion of human milk in infants' stomach their in vivo generation is most likely. Antimicrobially active peptides liberated from human milk proteins during enzymatic hydrolysis may play an important role in the host defense system of the newborn and of small infants.

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