Antibacterial Activity of Lactophorin, a Synthetic 23-Residues Peptide Derived from the Sequence of Bovine Milk Component-3 of Proteose Peptone

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ABSTRACT

A synthetic peptide of 23 residues corresponding to the carboxyterminal 113 to 135 region of component-3 of proteose peptone (PP3) has been investigated with regard to its antibacterial properties. This cationic amphipathic peptide that we refer to as lactophorin, displayed a growth-inhibitory activity against both gram-positive and gram-negative bacteria. For most of the strains tested, bacterial growth was observed in the presence of lactophorin except for *Streptococcus thermophilus*. In that case, lactophorin exhibited a minimum inhibitory concentration of 10 μ M and a minimum lethal concentration of 20 μ M. No hemolysis of human red blood cells was detected for peptide concentrations between 2 to 200 μ M, indicating that lactophorin would be noncytotoxic when used in this concentration range.

(Key words: bovine milk, component-3 of proteose peptone, antimicrobial activity, amphipathic peptide)

Abbreviation key: IC₅₀ = half inhibitory concentration, MIC = minimal inhibitory concentration, MLC = minimum lethal concentration, NMR = nuclear magnetic resonance, PP3 = component-3 of proteose peptone, RBC = red blood cell.

INTRODUCTION

Component-3 of proteose peptone (PP3), also called lactophorin, is a minor phosphoglycoprotein (135 residues) (Sorensen and Sorensen, 1993) found in bovine milk (accession number in SwissProt databank: P80195). Homologous proteins are characterized in milk of other species as camel (Beg et al., 1987), llama

(Cantisani et al., 1990), ewe, and goat (Sorensen et al., 1997; Lister et al., 1998). Up to now, PP3 has not been found in human milk. A 56% similarity is found between PP3 cDNA sequence (Groenen et al., 1995; Johnsen et al., 1995) and a gene coding for a glycosylation dependent adhesion molecule GlyCAM-1 in mice (Lasky et al., 1992) and rats (Dowbenko et al., 1993). GlyCAM-1 expression is observed in lymph nodes as well as in lung and mammary gland (Lasky et al., 1992), whereas PP3 is only expressed in the mammary gland of lactating cows (Groenen et al., 1995). In lymph nodes, GlyCAM-1 is indirectly involved in the immune response by acting as an endothelial cell surface ligand for L-selectin, a lymphocyte homing receptor (Girard and Almaric, 1998). This function requires the presence of sulfated and sialylated Lewis x antigen bound to the glycan moiety of GlyCAM-1 (Girard and Almaric, 1998), but sulfatation has not been demonstrated in mammary gland (Dowbenko et al., 1993). Despite the large homology observed between PP3 and GlyCAM-1, the exact function in vivo of bovine PP3 still remains unknown.

Structural data are becoming available to advance our knowledge of the function of PP3. Examination of the sequence of PP3 clearly indicates that the protein contains at least 2 distinct domains. The first one covers the N-terminal part (residues 1 to 97) and is largely negatively charged. This domain contains all the post-translational sites, i.e., 5 phosphorylation sites (Ser 29, 34, 38, 40, 46), two O-linked and one N-linked glycosylation sites (Thr 16 and 86, Asn 77, respectively) (Sorensen and Sorensen, 1993), modifications largely conserved for PP3 and GlyCAM-1. The second domain, from residue 98 to the C-terminal residue 135 is positively charged and displays a clear amphipathic character assuming an α -helical structure (Lasky et al., 1992; Campagna et al., 1999). The helical propensity of the 38-mer C-terminal fragment has been effectively demonstrated by a nuclear magnetic resonance (NMR) study in membrane-like environments (Bak et al.,

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2000). This amphipathic helical C-terminus seems to be also shared, although less conserved, by the homologous proteins of the GlyCAM-1 family (Lasky et al., 1992; Bak et al., 2000). The amphipathic domain explains the remarkable surface properties of PP3 and its ability to inhibit lipolytic activity (Girardet et al., 1993; Courthaudon et al., 1995).

To elucidate the biological function of the C-terminal domain of PP3, 2 peptides corresponding, respectively, to the 119 to 135 and 113 to 135 region have been synthesized. These peptides have been chosen as they preserve structural characteristics identical to the 98 to 135 domain. They exhibit similar charge ratio and same hydrophobic/hydrophilic sectors (according to the helical wheel projection) than the 98 to 135 region. The 2 peptides fold into a cationic amphipathic helix when placed in lipidic environment (Campagna et al., 1998, 2001), as the whole C-terminal domain does. The 17- and 23-mer peptides both interact with phospholipids, but only the 23-mer peptide can incorporate into planar lipidic bilayers by forming voltage-dependent channels (Campagna et al., 2001). The conductance levels indicate that channel formation may be achieved by association of 4 to 6 bundles of peptides according to the barrel-stave model.

Considering the pore-forming ability of the 113 to 135 C-terminal peptide of bovine PP3, it was conceivable that this peptide could interact with natural lipidic bilayers, such as bacterial membranes. Antimicrobial and hemolytic assays were therefore carried out using a synthetic peptide corresponding to peptide PP3 f(113 to 135). The results demonstrated that this peptide displayed inhibitory-growth activity against gram-positive and gram-negative bacteria but was inefficient to induce hemolysis of human red blood cells. The PP3 f(113 to 135) peptide was subsequently named "lactophorin."

MATERIALS AND METHODS

Materials

Lactophorin, the peptide corresponding to fragment f(113 to 135) of PP3 (sequence ¹NTVKETIKYLKSLFS-HAFEVVKT²³), was made commercially (Neosystem, Strasbourg, France) on a Synergy 432 peptide synthesizer (Perkin-Elmer Applied Biosystems, Foster City, CA) using the standard cycle for the 9-fluorenylmethoxycarbonyl strategy. Purification of the peptide was carried out by C₁₈ reversed-phase HPLC. The purified product was checked for sequence by amino acid analysis and mass spectrometry.

Antibacterial Assays

Bacterial strains were *Escherichia coli* ATCC 25922, *Salmonella* St Paul (DSV 29), *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Streptococcus thermophilus* NG40Z (CNRZ), and *Listeria innocua* (MC2, Ifremer).

Antibacterial activity of the peptide was evaluated by liquid growth inhibition assay in sterile 96-well polypropylene microplates using trypticase soy broth as growth medium, except for *Streptococcus thermophilus* for which M17 medium was used. The peptide was serially diluted in sterile Milli-Q water to give concentrations between 0.14 to 678 μ M (approx. 0.38 to 1840 μ g/mL). A standard inoculum of 16-h culture cells was added at a final concentration of 10⁵ cfu/mL. Control wells contained all the components except lactophorin. Experiments were done in duplicate. After incubation of the microplates for 24 h at 37°C, the absorbance at 620 nm of the assay mixture was determined and was corrected by subtraction of the absorbance due to the peptide alone in the culture medium. The minimal inhibitory concentration (MIC) was defined as the lowest peptide concentration inhibiting bacterial growth after incubation at 37°C for 24 h. It corresponded to peptide concentration at which there was less than 10% increase in the absorbance at 620 nm. The peptide concentration inducing a bacterial growth inhibition of 50% (IC₅₀) was determined spectrophotometrically when a complete inhibition could not be reached. For liquid growth experiments using *Strep. thermophilus*, after incubation for 24 h at 37°C, 0.1 mL of each well from the microplate was spread on a plate of M17 medium containing 1% agar (15 mL). The agar plates were examined daily for the formation of colonies. The minimal lethal concentration (MLC) was defined as the lowest peptide concentration at which no colony could be detected.

Hemolytic Assay

The hemolytic activity of lactophorin was determined using human red blood cells (RBC). A volume of 150 μ L of human blood was deposited into a conical tube containing 2 mL of Alsever's solution (19.3 mM sodium citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA, pH 7.0).

The RBC were then isolated by centrifugation (1000 $\times$ g, 5 m) and washed 3 times with an isotonic PBS consisted of a 35 mM sodium phosphate buffer pH 7.4, containing 0.154 M NaCl. The washed RBC were resuspended in 15 mL of PBS to reach a dilution of about 1% of the erythrocyte volume initially collected. Two hundred microliters of this RBC solution was intro-

Figure 1. Effect of lactophorin on growth of gram-positive bacteria. Bacterial growth was estimated by monitoring the absorbance at 620 nm after incubation at 37°C for 24 h. Trypticase soy broth was used as growth medium except for *Streptococcus thermophilus* for which M17 medium was used. (◇) *Listeria innocua* MC2, (□) *Strep. thermophilus* NG40Z, and (●) *Staphylococcus aureus* ATCC 25923. Experiments were done in duplicate.

Figure 2. Effect of lactophorin on growth of gram-negative bacteria. Bacterial growth was estimated by monitoring the absorbance at 620 nm after incubation at 37°C for 24 h. Trypticase soy broth was used as growth medium. (△) *Pseudomonas aeruginosa* ATCC 27853, (■) *Salmonella* St Paul DSV29, (×) *Escherichia coli* ATCC 25922. Experiments were done in duplicate.

duced in hemolysis tubes, and the volume was completed to 1 mL with the peptide suspended in PBS.

After 2 h of incubation at 37°C, the samples were centrifuged at $1000 \times g$ for 5 m and the absorbance of the supernatant was measured at 414 nm. Blank and 100% hemolysis controls were determined by replacing the peptide solution with PBS and 1% Triton X-100, respectively. The same experiment was also carried out with an incubation time extended to 24 h.

RESULTS

Antibacterial Activity

The antibacterial activity of bovine lactophorin was investigated against several gram-positive and gram-negative bacteria. The peptide was mainly active against gram-positive bacteria, with *Strep. thermophilus* being the most susceptible strain tested (Figure 1). An antimicrobial activity of the 23-mer peptide was also detected with gram-negative bacteria as *Salmonella* St Paul and *P. aeruginosa* (Figure 2). However, no activity was displayed against the *E. coli* strain used in this study even at high peptide concentration ($> 170 \mu\text{M}$). In a case in which the peptide was active, the bacterial growth was not completely inhibited even at peptide concentration above $170 \mu\text{M}$, except for *Strep. thermophilus* for which the MIC was of $10 \mu\text{M}$ (ca. $27 \mu\text{g/mL}$). The effect of lactophorin against this strain of

Streptococcus proved to be bactericidal with a MLC ($20 \mu\text{M}$) equal to twice the MIC. For the liquid growth assays in which no MIC could be determined, the peptide concentration inducing a bacterial growth inhibition of 50% (IC_{50}) was measured instead (Table 1). These values were compared with the MIC observed for antibacterial peptides from bovine milk when these data were indicated in the literature (Table 1). The antimicrobial activities against *Strep. thermophilus* of lactophorin and the CN peptide $\alpha_{s2}\text{-CN f}(183 \text{ to } 207)$ were of the same magnitude and 5-fold higher than the activity of the casein peptide $\alpha_{s2}\text{-CN f}(164 \text{ to } 179)$. However, this comparison must be taken with care, as the bacterial strains used in the 2 studies were not the same. The antibacterial activity spectrum of lactophorin was broad with an inhibitory-growth effect on gram-positive as well as on gram-negative bacteria, but this activity remained moderate as the bacterial growth was maintained (except for *Strep. thermophilus*) (Figures 1 and 2).

It can be noted that the peptide concentrations leading to a bacterial growth inhibition were much higher than the effective peptide concentration that promotes channel formation in planar lipid bilayers (concentration in the range of 20 nM , Campagna et al., 2001). For concentrations above $170 \mu\text{M}$ (ca. $460 \mu\text{g/mL}$), a significant dose-dependent absorbance of the peptide suspended in trypticase soy broth was observed at 620 nm, whereas the absorbance of the peptide in aqueous

Table 1. Antibacterial activity of lactophorin and of known antibacterial peptides from bovine milk. (Experiments made with lactophorin in the present work were done in duplicate.)

Peptide	Length	MIC ¹ (μ M)	IC ₅₀ ² (μ M)	Bacterial strain
Lactophorin	23	10 >300 ³ >300 ³ >300 ³ >300 ³	45 75 40 110 >340 ³	<i>Streptococcus thermophilus</i> (NG40Z) <i>Staphylococcus aureus</i> (ATCC 25923) <i>Listeria innocua</i> (MC 2) <i>Salmonella</i> St Paul (DSV 29) <i>Pseudomonas aeruginosa</i> (ATCC 27853) <i>Escherichia coli</i> (ATCC 25922)
Other peptides from bovine milk				
α_{s2} -CN f(164–179) ⁴	16	50 50 25		<i>Strep. thermophilus</i> Rs <i>L. innocua</i> <i>E. coli</i> (ATCC 25922)
α_{s2} -CN f(183–207) ⁴	25	8 16 16		<i>Strep. thermophilus</i> Rs <i>L. innocua</i> <i>E. coli</i> (ATCC 25922)
Lactoferrin B ⁵	25	2 3 2		<i>Staph. aureus</i> (JCM-2151) <i>P. aeruginosa</i> (IFO-3446) <i>E. coli</i> (IID-861)

¹MIC was defined as the lowest concentration of peptide required to inhibit bacterial growth.

²For experiments where bacterial growth was not completely inhibited, an IC₅₀ was determined that corresponds to the peptide concentration inducing a bacterial growth inhibition of 50%.

³> Denotes no inhibition detected at the concentration indicated.

⁴Recio and Visser, 1999.

⁵Bellamy et al., 1992.

solution was negligible at the same wavelength (Figure 3). In addition, absorbance of lactophorin dissolved in Milli-Q water was also recorded at 275 nm as a function of peptide concentration to control the absence of peptide precipitation (Figure 3) so that peptide concentrations could be determined using the M extinction coefficient of tyrosine ($\varepsilon_{275\text{nm}} = 1420 \text{ M}^{-1}\cdot\text{cm}^{-1}$). The absorbance at 275 nm was proportional to the peptide

concentration, indicating a monomeric state of lactophorin in water for all the concentrations tested.

Hemolytic Assay

Hemolytic activity measurements using human RBC were undertaken to study the interaction of the synthetic 23-mer peptide with eukaryotic cell membrane and the eventual toxicity of lactophorin. The RBC were incubated at 37°C with the peptide at a concentration range from 2 to 200 μ M. No significant hemolytic activity was detected even after extending the incubation time from 2 h to 24 h (data not shown). These results indicate that lactophorin would be noncytotoxic at concentrations lower than 0.2 mM (approximately 20 times higher than the MIC observed with *Strep. thermophilus*).

DISCUSSION

The liquid growth inhibition assays achieved on different bacterial strains reveal an antimicrobial potency of lactophorin. The antibacterial activity displayed by the 23-mer peptide could appear low toward what was expected considering its pore-forming activity at the nanomolar range (Campagna et al., 2001). However, no formal relationship can be established between the pore-forming and antibacterial peptide concentrations. Indeed, in planar lipid bilayer experiments, high peptide/lipid ratios were used, and the bilayers were made

Figure 3. Absorbances of lactophorin at 620 and 275 nm (insert). Peptide was dissolved in (■) Milli-Q water or in (○) trypticase soy broth. Measurements were recorded at room temperature.

of synthetic pure phospholipids (Campagna et al., 2001), whereas biological membranes are far more complex. The relatively high lactophorin concentrations required in some cases to limit bacterial growth could be explained by alteration of the peptide structure by bacterial proteases excreted in the medium or present at the cell surface (Andreu and Rivas, 1998). This is shown with a protease of *Salmonella* (Tossi et al., 2000), which presents an activity similar to magaininase, an enzyme involved in the degradation of magainins as well as other antibacterial peptides (Groisman, 1994).

The linearity of the relationship between peptide concentration and its absorbance at 275 nm showed that the peptide was present in a monomeric form in water. The absorbance at 620 nm (the wavelength used to follow bacterial growth) of the peptide aqueous solution was close to zero as expected (no chemical groups from a peptide absorb at this wavelength). However, when lactophorin was suspended in the culture medium (trypticase soy broth), a dose-dependent absorption was observed at 620 nm for concentrations of the peptide above 170 μM . Interestingly, this concentration constituted a threshold in experiments at which the peptide was unable to completely inhibit the bacterial growth. This phenomenon could be explained by formation of large soluble aggregates of peptide molecules interacting with components of the culture medium and inducing light scattering. These nonproductive interactions would occur to the detriment of interactions between lactophorin and bacterial surfaces. However, we did not test in this study other culture growth media that could allow a better solubilization of high concentration of lactophorin (>170 μM) since antimicrobial activity is generally considered efficient only for peptide concentration lower than 100 μM .

From the investigations of potential hemolytic activity, it appears that lactophorin was not able to induce the lysis of human red blood cells. The variation of charge between the outer leaflet of mammalian cells (mainly composed of zwitterionic phospholipids), and the bacterial anionic membranes seems not sufficient to explain the absence of hemolytic activity, as the peptide also generates channel formation in bilayer made with zwitterionic phospholipids (Campagna et al., 2001). The considerably lower transmembrane potential of animal cells has been proposed as a possible explanation for the absence of hemolytic activity observed for some antibacterial peptides (Tossi et al., 2000). For example, the value of the electrical potential ($\Delta\psi$) across the cell membrane is in the range from -100 to -160 mV for *Staph. aureus* (depending on its physiological state) (Vinnikov et al., 1989), whereas the transmembrane potential of human red blood cells is only of -10.1 mV $\pm$ 1.8 mV (Zavodnik et al., 1997). Interestingly, the pore-

forming activity of the 23-mer peptide is voltage-dependent and the minimal voltage value required to observe an active transmembrane orientation of the peptide molecules is -40 mV in zwitterionic lipids (Campagna et al., 2001). Therefore, even if the peptide would be able to cross the glycocalyx of red blood cells and reach the lipidic membrane, it would probably not form channel because transmembrane voltage was too low.

CONCLUSIONS

In summary, lactophorin presents an inhibitory-growth activity against both gram-negative and gram-positive bacteria, but no hemolytic activity in the peptide concentration range tested ($\leq 200 \mu\text{M}$). For the bacteria tested in this study, a 100% bacterial growth inhibition was only observed with nonpathogenic *Strep. thermophilus* strain. However, the low MIC (10 μM) and MLC (20 μM) values observed in this case are promising. Therefore, we will further investigate the lactophorin antibacterial activity against other pathogenic strains. Although lactophorin displayed a low antibacterial activity compared with other peptides, this is the first report of an antimicrobial peptide from bovine milk PP3 or, in more general terms, from the PP3 protein family.

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