# Antibacterial Peptides of Bovine Lactoferrin: Purification and Characterization

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## ABSTRACT

Three peptides with antibacterial activity toward enterotoxigenic *Escherichia coli* have been purified from a pepsin digest of bovine lactoferrin. All peptides were cationic and originated from the N-terminus of the molecule in a region where a bactericidal peptide, lactoferricin B, had been previously identified. The most potent peptide, peptide I, was almost identical to lactoferricin B; the sequence corresponded to residues 17 to 42, and the molecular mass was 3195 as determined by mass spectrometry. A second, less active peptide, peptide II, consisted of two sequences, residues 1 to 16 and 43 to 48 (molecular mass of 2673), linked by a single disulfide bond. The third peptide, peptide III, also a disulfide-linked heterodimer, corresponded to residues 1 to 48 (molecular mass of 5851), cleaved between residues 42 and 43. Peptides I and II displayed antibacterial activity toward a number of pathogenic and food spoilage microorganisms, and peptide I inhibited the growth of *Listeria monocytogenes* at concentrations as low as 2 $\mu M$. Bacterial growth curves showed that bactericidal effects of peptides I and II were observable within 30 min of exposure. The results confirmed and extended those of earlier studies suggesting that the bactericidal domain of lactoferrin was localized in the N-terminus and did not involve iron-binding sites.
(**Key words**: lactoferrin, pepsin digest, antibacterial peptides)

**Abbreviation key**: **Lf** = lactoferrin, **TFA** = trifluoroacetic acid.

## INTRODUCTION

Lactoferrin (**Lf**) is an iron-binding glycoprotein that is present in mammalian exocrine secretions and in specific granules of polymorphonuclear leukocytes (3, 16). The concentration of Lf in the mammary gland varies dramatically with its functional status. Mature bovine milk contains 0.1 to 0.3 mg/ml, and colostrum contains 2 to 5 mg/ml (7). During involution of the bovine mammary gland, the level may reach 20 to 30 mg/ml (7). Sanchez et al. (21) have reported Lf concentrations of 1.2 mg/ml in milk samples from mastitic cows compared with Lf concentrations of 0.09 mg/ml in milk from healthy cows.

The protein has bacteriostatic and bactericidal properties attributed to its ability to chelate iron (8, 24) or to bind to the bacterial surface (2, 9). Studies (11, 12) have shown that Lf damages the outer membrane of Gram-negative bacilli, causing the release of lipopolysaccharides that sensitize the cell to antibiotic action. The finding by Tomita et al. (22) that pepsin digestion of bovine Lf produces potent bactericidal peptides has led to the identification of a bactericidal domain on the protein (5, 23). The active peptide, lactoferricin B, is in a region of the molecule that is distinct from the iron-binding sites, which provides strong evidence for a bactericidal mechanism that is independent of iron. The peptide is capable of binding lipopolysaccharide molecules and releasing them from the outer membrane of Gram-negative bacteria in a manner similar to that of the native Lf molecule (25).

In a previous study, Dionysius et al. (10) reported on the antibacterial activity of several forms of bovine Lf toward enterotoxigenic *Escherichia coli*. Bactericidal activity was evident for both apo-Lf and zinc-saturated Lf; charge as well as structural elements were implicated in the mechanism of action. Following reports that a single cationic peptide, lactoferricin B, was solely responsible for the antimicrobial activity of bovine Lf (23), we undertook studies to identify other peptides derived from Lf that could contribute to its activity. We report here the finding that a peptide almost identical to lactoferricin B and two other cationic peptides from the N-terminus of Lf, generated by peptic cleavage, contribute to the bactericidal activity. One of these peptides has a sequence that previously has not been reported to be antibacterial.

## MATERIALS AND METHODS

### Pepsin Digestion of Lf

Bovine Lf was purified from Cheddar cheese whey by cation-exchange chromatography using the method of Law and Reiter (14). A 5% (wt/vol) aqueous solution was adjusted to pH 2.5 with 2$N$ HCl and digested with 0.15% (wt/vol) porcine pepsin (Sigma Chemical Co., St Louis, MO) for 4 h at 37°C. The reaction was terminated by heating at 80°C for 15 min, and the solution was adjusted to pH 7.0 with 1$N$ NaOH. The digest was then centrifuged at $17,000 \times g$ for 15 min in a refrigerated centrifuge (Sorvall RC-5C; Du Pont Co., Wilmington, DE), and the supernatant was lyophilized.

### Purification of Peptides

*Ion-exchange chromatography.* The Lf digest was dissolved in 0.05 $M$ phosphate buffer, pH 7.5 (load buffer), and loaded onto a column of S-Sepharose Fast Flow (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in the same buffer. The column was washed with load buffer, and bound peptides were then eluted with load buffer containing 1 $M$ NaCl. The absorbance of the column effluent was monitored at 280 nm.

*Reverse-phase HPLC.* The 1 $M$ NaCl eluate from ion-exchange chromatography was desalted by HPLC on a C18 reverse-phase column (2.2 cm i.d. × 25 cm; Vydac, Hesperia, CA). The column was equilibrated with 0.1% trifluoroacetic acid (**TFA**; solvent A), and the sample, adjusted to 0.1% TFA, was loaded at 5 ml/min. The peptide material was washed with solvent A until the baseline returned to zero and then was eluted with a 20:80 mixture of solvent A and solvent B (90% acetonitrile in 0.09% TFA) at the same flow rate. The absorbance of the column eluent at 220 and 280 nm was monitored continuously. Desalted peptide material was lyophilized and stored at –20°C.

The peptides were fractionated by HPLC using a preparative C18 reverse-phase column (2.2 cm i.d. × 25 cm; Vydac). Sample (200 mg dissolved in solvent A) was loaded onto the column previously equilibrated with solvent A, and the column was washed extensively with solvent A followed by a 5-min wash with an 80:20 mixture of solvent A and solvent B. Peptides were eluted using a gradient of solvent B from 20 to 50% over 35 min at a flow rate of 5 ml/min. Fractions were collected at 30-s intervals and were pooled according to the retention times of separated peptides.

Pooled peptide peaks were tested for antibacterial activity, and those with high activity were further purified using an analytical C18 column (0.46 cm i.d. × 25 cm; Vydac). The methods used were similar to those described, except that a gradient of solvent B from 20 to 35% (peptides I and II) or 25 to 40% (peptide III) was employed to fractionate the peptides (10-mg load), and the flow rate was 1 ml/min. Purified peptides were lyophilized and stored at –20°C.

### Peptide Characterization

*Analytical HPLC.* Samples of purified peptides were analyzed by reverse-phase HPLC using the analytical column and method described previously, except that a gradient of solvent B from 20 to 50% was employed.

*Mass spectrometry.* The mass of the purified peptide was determined by ionspray mass spectrometry (PE SCIEX API 111 triple quadrupole mass spectrometer; PE SCIEX Instruments, Thornhill, ON, Canada). The mass spectrometer was operated in the positive ion mode (ionspray voltage, +5 kV). A sample (5 µl of 1 mg/ml in 0.1% TFA) was injected into the solvent delivery line at 30 µl/min (solvent A, 0.05% TFA; solvent B, 90% acetonitrile in 0.05% TFA; and solvent mixture, 50% solvent B). Typical scanning conditions were m/z of 300:2400 in 5 s with a scan step of 0.5 atomic mass units. Spectra were collected in the multichannel averaging mode at an orifice potential set between 60 and 80 V. Molecular masses of charged species were calculated using the HyperMass software (PE SCIEX Instruments).

*Amino acid analysis.* Peptides were subjected to gas phase hydrolysis with 6$N$ HCl at 121°C for 24 h in vacuo using a Pico-Tag workstation (Waters Associates, Milford, MA). Hydrolysates were dried, neutralized, and derivatized with phenylisothiocyanate and then chromatographed with a multisolvent delivery system (model 600; Waters Associates) using 0.14 $M$ sodium acetate, pH 7.5, and acetonitrile gradient elution. The column was monitored at 254 nm, and individual amino acids were assigned by comparison with an amino acid standard (Pierce Chemical Co., Rockford, IL).

*Peptide sequence analysis.* The N-terminal amino acid sequencing of purified peptides was performed on a protein sequencer (model 470A) with an on-line PTH analyzer (model 120A; Applied Biosystems Inc., Foster City, CA). For sequencing of a heterodimer crosslinked by a disulfide bond, the sample was first subjected to reductive alkylation with 4-vinylpyridine. The alkylated peptides were sepa-

rated by reverse-phase HPLC on a microbore C8 column (1.0 mm i.d. × 50 mm) using a linear gradient (90 min; 100 μl/min) from solvent A to solvent B; solvent A was 0.1% TFA, and solvent B was 0.09% TFA in 60% acetonitrile. The column effluent was monitored at 215, 254, and 280 nm. Peptides were collected into Eppendorf tubes and loaded onto polybrene-coated glass fiber discs for automated sequencing.

## Bacteria and Culture Conditions

Enterotoxigenic *E. coli* isolates from piglets with neonatal or postweaning scours were used as routine test microorganisms in this study. *Pseudomonas fluorescens* was isolated from raw milk; *Bacillus cereus* ACM 446 was obtained from the University of Queensland (Brisbane, Australia), and *Salmonella salford* IMVS 1710 was obtained from the Institute of Medical and Veterinary Science (Adelaide, Australia). *Listeria monocytogenes* NCTC 7973 and *Staphylococcus aureus* NCTC 6571 were from the National Collection of Type Cultures (London, England). All bacterial isolates were maintained on slants of peptone and yeast extract (Difco Laboratories, Detroit, MI) agar at 4°C and subcultured every 2 mo to ensure viability. Working inocula for antibacterial assays were prepared by subculture of isolates from agar slants into Todd-Hewitt broth (Becton Dickinson and Co., Cockeysville, MD) and incubated at 37°C for 16 h. Cultures of *P. fluorescens* were grown at 30°C.

## Antibacterial Assays

A modification of the microassay of Nonnecke and Smith (19) was used to test antibacterial activity. Briefly, 0.5 ml of a working inoculum of bacterial culture was added to 10 ml of Todd-Hewitt broth and incubated in a shaking water bath for 6 h at 37°C. The culture was then diluted in 0.1% peptone to a final concentration of approximately $1 \times 10^4$ cfu/ml and used for inoculation of the microassay.

Assays were carried out in sterile 96-well tissue culture plates (Linbro; Flow Laboratories Inc., McLean, VA). The growth medium (pH 6.8) consisted of 1% peptone (Difco Laboratories), 0.05% yeast extract (Difco Laboratories), and 1% glucose. To each well was added 100 μl of growth medium, 25 μl of peptide in deionized water, and 5 μl of bacterial inoculum. Control assays contained all components except peptide. Plates were covered with sterile lids and incubated at 37°C for 20 h, except for *P. fluorescens* (30°C for 20 h) and *L. monocytogenes*

(37°C for 40 h). The absorbance of the assay mixture at 620 nm was determined (Titertek Multiskan plate reader; Flow Laboratories Inc.). The MIC was defined as the lowest concentration of peptide or protein that gave no increase in absorbance at 620 nm following incubation. Purified peptides were tested at concentrations from 3.1 to 400 μg/ml, and the Lf concentration varied from 125 to 4000 μg/ml. The effect of cations on the antibacterial activity of peptides was tested at concentrations from 10 to 100 m*M* for monovalent cations ($Na^+$ and $K^+$) and 2.5 to 5 m*M* for divalent cations ($Mg^{2+}$). All assays were performed in triplicate.

Growth curves for *E. coli* strain 1 incubated with or without peptide were obtained by incubation of bacterial cultures at 37°C in tubes containing growth medium (5 ml), peptide (1.25 ml in deionized water), and bacterial suspension ($2.5 \times 10^3$ cfu in 0.25 ml of 0.1% peptone). Samples were taken at selected times over 4 h and serially diluted with sterile 0.1% peptone. Viable counts were determined by plating selected dilutions on plate count agar (Oxoid Ltd., Basingstoke, Hampshire, England). Plates were incubated at 37°C for 48 h, and bacterial colonies were counted visually. All assays were performed in duplicate, and the means are reported.

# RESULTS

## Purification of Antibacterial Peptides

The digestion of bovine Lf with porcine pepsin yielded peptides with molecular masses <10,000 Da, and SDS-PAGE and analytical HPLC results showed the absence of residual native Lf in the digest (data not shown). Cation-exchange chromatography of the digest on S-Sepharose Fast Flow at pH 7.5 effected a fourfold purification of the antibacterial peptides; all activity was contained in the 1 *M* NaCl eluate. Subsequent purification procedures employed reverse-phase HPLC techniques, and three peptides with antibacterial activity toward *E. coli* were purified to homogeneity. The HPLC profiles of the purified peptides, together with that of the 1 *M* NaCl eluate from cation-exchange chromatography, are shown in Figure 1. Peptides I and II were the two major components bound to S-Sepharose Fast Flow (Figure 1).

## Characterization of Peptides

Purified peptides were subjected to mass spectrometry, N-terminal amino acid sequencing, and amino acid analysis in order to determine their location in the native Lf molecule. Peptide I has a mass of 3195

as determined by mass spectrometry. The amino acid sequence corresponded exactly to residues 17 to 42 of bovine Lf (13, 20), and the amino acid data (results not shown) confirmed the sequence assignment. The molecular mass that was calculated from the se-

quence data was consistent with that determined by mass spectrometry; the latter indicated the presence of an internal disulfide bridge between residues 19 and 36. This peptide was almost identical to the bactericidal peptide, lactoferricin B, that was described by Bellamy et al. (5), except for the presence of an additional alanine (residue 42) on the C-terminus.

Initial sequencing data for peptide II suggested two N-termini, and subsequent reductive alkylation, chromatographic separation, and sequencing confirmed the presence of two peptides linked by a disulfide bond. This heterodimer corresponded exactly to residues 1 to 16 and 43 to 48 of bovine Lf (13, 20), and the two peptides were linked by a disulfide bridge at residues 9 and 45. Mass spectrometry showed that the native peptide had a mass of 2673, which was consistent with the mass calculated from the sequence. The identity of the two peptides was also confirmed by mass spectrometry of the separated alkylated peptides. Results of amino acid analysis were consistent with sequence data.

Peptide III had a molecular mass of 5851 as determined by mass spectrometry, and N-terminal amino acid sequencing revealed two N-termini in equimolar concentrations, a result indicative of two peptides linked by a disulfide bond. Sequence assignment for the first five amino acids corresponded to residues 1 to 5 (APRKN) and 43 to 47 (LECIR) of bovine Lf (Figure 2) (13, 20). The results of mass spectrometry and amino acid sequencing revealed that peptide III corresponded to residues 1 to 48 of bovine Lf and that the two peptides (1 to 42, 43 to 48) were linked by a disulfide bridge at residues 9 and 45. A schematic representation of the primary structures of the three peptides is shown in Figure 2.
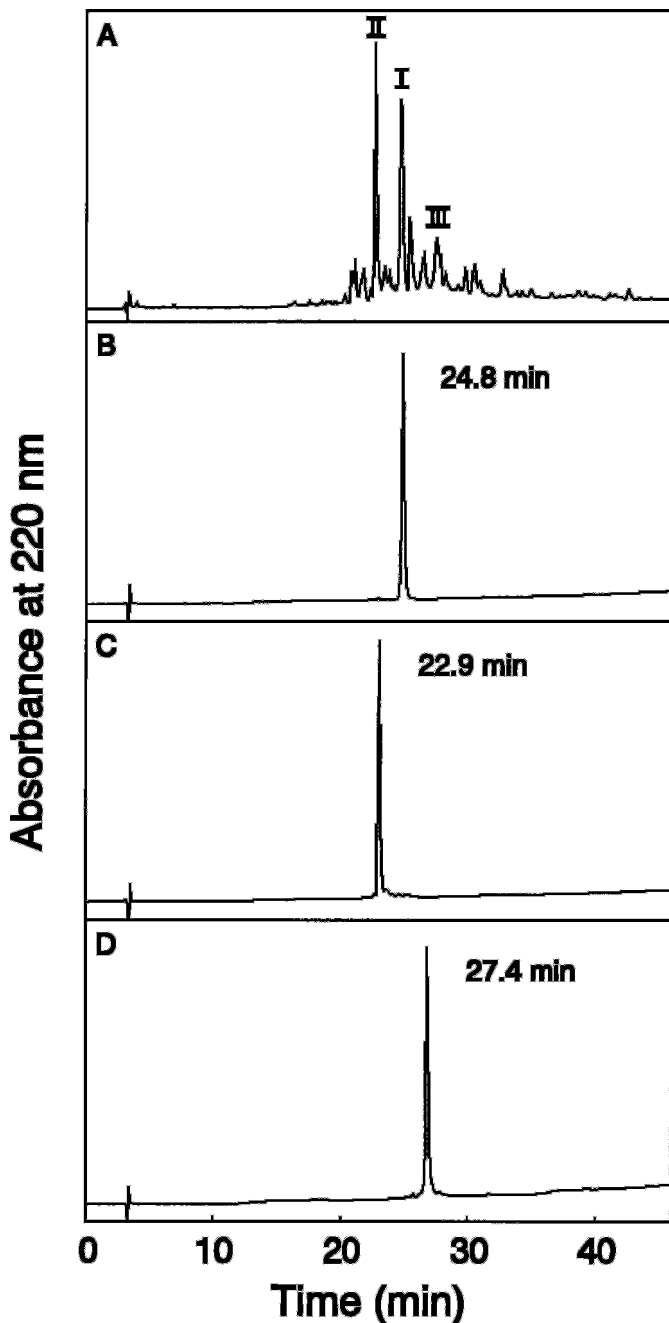


Figure 1. Reverse-phase HPLC chromatograms of peptides generated by pepsin digestion of bovine lactoferrin: 1 $M$ NaCl eluate from S-Sepharose Fast Flow chromatography (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) (A) and purified antibacterial peptides I (B), II (C), and III (D), respectively.

## Antibacterial Activity of Purified Peptides

The antibacterial activity of the purified peptides toward *E. coli* strain 1 is shown in Table 1. On a molar basis, peptide I was three times as effective as native Lf; the activity of peptides II and III was much weaker. Peptide III, which contained the sequence of the most active peptide I, had an activity comparable with native Lf.

Antibacterial activity of peptides I and II toward a variety of Gram-positive and Gram-negative bacteria is shown in Table 2. Peptide I was consistently more active than peptide II and showed broad activity. In agreement with the results of Bellamy et al. (4) for lactoferricin B, *L. monocytogenes* was highly sensitive to peptide I, and *P. fluorescens* was relatively resistant. Peptide II displayed weak antibacterial activity against all bacteria tested.

The bactericidal activity of peptides I and II toward *E. coli* strain 1 is shown in the growth curves of Figure 3. At concentrations that were twice the MIC for the respective peptides, loss of viability was significant after incubation for 30 min at 37°C. Control cultures with no added peptide continued to grow under these conditions. Insufficient purified material precluded the testing of peptide III in these experiments.

The effect of monovalent and divalent cations on the inhibitory activity of peptide I was studied using two strains of *E. coli* (Table 3). In both strains, the addition of cations diminished the bactericidal activity of the peptide; divalent ions ($Mg^{2+}$) were much more effective than monovalent ions ($Na^+$ and $K^+$). Some differences in sensitivity between bacterial strains in the presence of both monovalent and divalent cations were also evident. When *E. coli* strains 1 and 2 were tested for their sensitivity to inhibition by Lf, strain 1 was more sensitive to inhibition than was
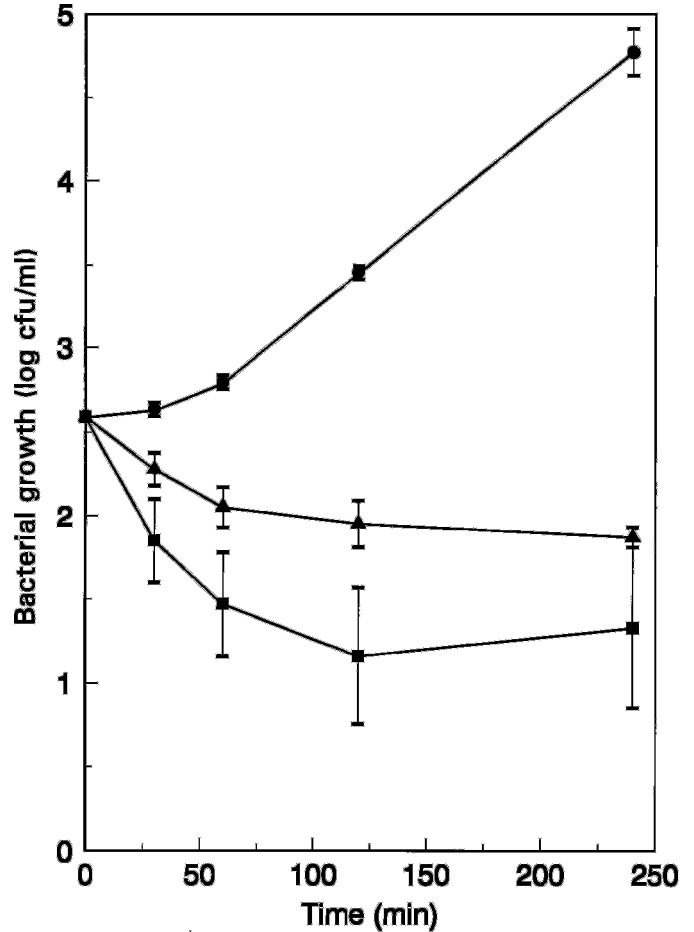


Figure 3. Effect of lactoferrin peptide I (25 μg/ml; ■) and peptide II (400 μg/ml; ▲) on the growth of *Escherichia coli* strain 1 at 37°C. The control (●) contained no added peptide. Each data point shown is the mean (±SEM) from triplicate experiments.
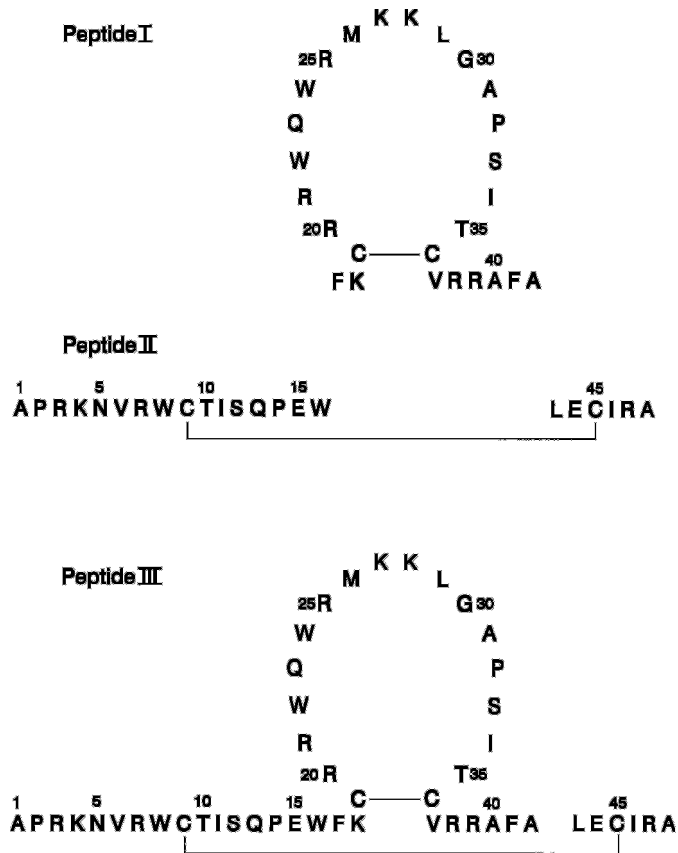


Figure 2. Primary sequences of antibacterial peptides generated by pepsin digestion of bovine lactoferrin. Residue numbers indicate sequence position in bovine lactoferrin (13, 20), and disulfide bonds are shown as lines.

strain 2 (MIC, 1000 and 2000 μg/ml, respectively). This difference in sensitivity was also observed when peptide I was used as the inhibitory agent (Table 3).

## DISCUSSION

The present study characterizes the peptides that contributed to the antibacterial mechanism of bovine Lf. All three active peptides were contained in the first 48 residues of the N-terminus, all were basic peptides (theoretical isoelectric points ranging from 9.3 to 12.4), and none were involved in binding iron (15). In the studies of Bellamy et al. (5), the bactericidal domain of bovine Lf was identified as a single peptide from the N-terminus of the molecule. The peptide, lactoferricin B, corresponded to residues 17 to 41 of the native protein, and its sequence (and molecular mass) was assigned solely on the results of

TABLE 1. Antibacterial activity of bovine lactoferrin (Lf) and its peptides toward *Escherichia coli* strain 1.

| Lf Derivative | MIC[1] | |
|---|---|---|
| | ($\mu$g/ml) | ($\mu M$) |
| Native Lf | 1000 | 13 |
| Pepsin digest | 250 | . . . |
| S-Sepharose Fast Flow,[2] | | |
| 1 *M* NaCl eluate | 100 | . . . |
| Peptide I | 12.5 | 4 |
| Peptide II | 200 | 75 |
| Peptide III | 100 | 17 |

[1]Defined as the lowest concentration of protein or peptide that gave no increase in absorbance at 620 nm after incubation at 37°C for 20 h. Native Lf and pepsin digest were tested in serial dilution from 2000 to 125 $\mu$g/ml; all other derivatives were tested from 400 to 6.3 $\mu$g/ml.

[2]Pharmacia LKB Biotechnology AB (Uppsala, Sweden).

amino acid sequence analysis. Peptide I described in our study had an additional residue on the C-terminus compared with lactoferricin B, but our studies otherwise confirmed that this peptide was the major contributor to bactericidal activity. We have performed independent determinations of the structure of peptide I (mass spectrometry, N-terminal sequencing, and amino acid analysis), and lactoferricin B is probably identical to this peptide because similar conditions were employed in the pepsin digestion of Lf. Additional studies, such as mass spectral analysis and amino acid analysis of lactoferricin B, are needed to confirm this assumption.

Peptide III shares structural homology with human lactoferricin [lactoferricin H; (5)]; both peptides contain two subfragments linked by a disulfide bridge, and both have similar origins in the native molecule (1 to 47 and 1 to 48 for human and bovine peptides, respectively). Peptide III was four times less active than peptide I, even though peptide III contained the complete sequence of peptide I. Bellamy et al. (5) found that the activity of lactoferricin H was nine times lower than that of lactoferricin B. It may be speculated that, in the larger peptides (lactoferricin H and peptide III), sequences flanking the active loop (residues 20 to 37 and 19 to 36 in human and bovine peptides, respectively) hinder effective interaction with the bacterial surface. With peptide I, a molecule with smaller flanking sequences, such interference would be minimized.

Peptide II contained a sequence that has not previously been reported to be part of the bactericidal domain of bovine Lf. This peptide flanked the active loop region of peptide I and, although not as potent (or as basic) as peptide I, peptide II clearly demonstrated a bactericidal effect toward *E. coli*. Care has been taken to exclude the possibility of contamination of the peptide with more active peptides, and mass spectrometry and HPLC confirmed the absence of the latter in the preparations used to define antibacterial activity. Therefore, the region of the native molecule that is involved in the antibacterial mechanism is larger than previously thought, and further studies may reveal that this secondary peptide acts to disrupt membranes in a manner similar to lactoferricin B. Gram-negative bacteria exposed to the latter peptide showed immediate development of electron-dense membrane blisters, providing evidence of damage to the outer membrane (25).

In a previous study, Dionysius et al. (10) observed marked differences in sensitivity between strains of enterotoxigenic *E. coli* to inhibition by Lf. When a much smaller molecule, such as the peptide I characterized in this study, was used as the bactericidal agent, strain differences were still evident. This finding suggests that accessibility of the active component to target sites on the bacterial cell surface is not a major factor in the antibacterial mechanism of Lf and

TABLE 2. Antibacterial activity of lactoferrin peptides toward various bacteria.

| Bacteria | MIC[1] | | | |
|---|---|---|---|---|
| | Peptide I | | Peptide II | |
| | ($\mu$g/ml) | ($\mu M$) | ($\mu$g/ml) | ($\mu M$) |
| *Escherichia coli* | 12.5 | 4 | 200 | 75 |
| *Pseudomonas fluorescens* | 50 | 16 | 400 | 150 |
| *Salmonella salford* | 25 | 8 | 200 | 75 |
| *Listeria monocytogenes* | 6.3 | 2 | 200 | 75 |
| *Bacillus cereus* | 25 | 8 | 400 | 150 |
| *Staphylococcus aureus* | 50 | 16 | 400 | 150 |

[1]Defined as the lowest concentration of peptide that gave no increase in absorbance at 620 nm under defined incubation conditions. Peptide I was tested in serial dilution from 100 to 3.1 $\mu$g/ml; peptide II was tested from 400 to 50 $\mu$g/ml.

TABLE 3. Antibacterial activity of peptide I from lactoferrin toward *Escherichia coli* in the presence of salts.

| | MIC[1] | |
|---|---|---|
| Addition | E. coli strain 1 | E. coli strain 2 |
| | ($\mu$g/ml) | |
| None | 12.5 | 25 |
| NaCl, m*M* | | |
| 10 | 25 | 25 |
| 25 | 25 | 50 |
| 50 | 50 | 100 |
| 100 | >100 | >100 |
| KCl, m*M* | | |
| 10 | 25 | 25 |
| 25 | 25 | 50 |
| 50 | 50 | 100 |
| 100 | >100 | >100 |
| MgCl$_2$, m*M* | | |
| 2.5 | 25 | 50 |
| 5.0 | 50 | 100 |

[1]Defined as the lowest concentration of peptide that gave no increase in absorbance at 620 nm after incubation at 37°C for 20 h. The peptide was tested in serial dilution from 200 to 6.3 $\mu$g/ml.

peptides derived from Lf. However, differences in the composition of the components of the bacterial outer membrane (e.g., lipopolysaccharides and their associated stabilizing cations) may influence resistance to membrane-disrupting agents such as Lf and cationic peptides. Naidu et al. (17) correlated the binding of Lf to the porin proteins of the outer membrane with antimicrobial efficacy and suggested that the polysaccharide moiety of lipopolysaccharides may block the specific interaction between Lf and porin. It is yet to be established whether cationic Lf peptides have a similar interaction with the porins of the bacterial outer membrane.

Bellamy et al. (4, 6) have shown that divalent cations reduce the cell binding of radiolabeled lactoferricin B to *E. coli* and *Bacillus subtilis* and also reduce its antibacterial effectiveness. At concentrations of Mg$^{2+}$ that were similar to those used by Bellamy et al. (4, 6), we confirmed that divalent cations decreased the activity of peptide I toward both *E. coli* strains. Small but consistent differences in sensitivity between the strains probably reflected variations in anionic cell wall components such as lipopolysaccharides and phospholipids (18).

The concentration of Lf rises sharply during infection of the mammary gland (21). In addition, polymorphonuclear leukocytes containing hydrolytic enzymes enter the gland to fight the infection. In the microenvironment of the phagosome, conditions may suit the cleavage of Lf by aspartic proteases similar to those that have been shown to yield lactoferricin (25). Hence, in the mastitic mammary gland, significant quantities of bactericidal peptides may be released to assist in fighting the infection. Lactoferrin levels are also elevated in colostrum (7). It is probable that ingested Lf protects the newborn calf from gastrointestinal infection by presenting higher concentrations of the intact protein and by favoring the generation of antibacterial peptides in the stomach. These two examples, one in the maternal mammary gland and the other in the neonatal gut, show how cationic bactericidal peptides may be generated to assist in protecting against a microbial challenge.

The susceptibility of a wide range of pathogenic and food spoilage microorganisms to the cationic peptides of Lf has been confirmed in this study. Enterotoxigenic *E. coli*, food spoilage bacteria (*P. fluorescens*), and food pathogens (*L. monocytogenes*, *B. cereus*, *Sal. salford*) were all inhibited by peptide I at relatively low concentrations. The development of large-scale procedures for ion-exchange purification would appear relatively straightforward for these highly basic peptides, which therefore offer potential as natural food preservatives or as antibacterial agents in medical and veterinary applications.

## CONCLUSIONS

The findings of this study add to the growing body of evidence that the bactericidal effects of Lf are due to specific cationic peptides from the N-terminal region. It may be speculated that two antibacterial mechanisms exist within Lf: bacteriostatic activity associated with iron chelation via the two iron-binding sites and bactericidal activity restricted to an exposed domain, which becomes buried and consequently ineffective upon iron binding (1). Following in vivo digestion of Lf by proteolytic enzymes, the three peptides that were characterized in this study may be generated by the action of pepsin and provide continued protection against a wide range of Gram-positive and Gram-negative bacteria. Evidence for in vivo production of antibacterial peptides has been provided by Tomita et al. (23), who isolated lactoferricin B from the gastrointestinal contents of rats fed a diet containing bovine Lf.

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