ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1997, p. 54–59 Vol. 41, No. 1
0066-4804/97/$04.00+0

Antibacterial Activity of Bovine Lactoferrin-Derived Peptides

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Received 30 April 1996/Returned for modification 31 May 1996/Accepted 11 October 1996

Several peptides sharing high sequence homology with lactoferricin B (Lf-cin B) were generated from bovine lactoferrin (Lf) with recombinant chymosin. Two peptides were copurified, one identical to Lf-cin B and another differing from Lf-cin B by the inclusion of a C-terminal alanine (lactoferricin). Two other peptides were copurified from chymosin-hydrolyzed Lf, one differing from Lf-cin B by the inclusion of C-terminal alanyl-leucine and the other being a heterodimer linked by a disulfide bond. These peptides were isolated in a single step from chymosin-hydrolyzed Lf by membrane ion-exchange chromatography and were purified by reverse-phase high-pressure liquid chromatography (HPLC). They were characterized by N-terminal Edman sequencing, mass spectrometry, and antibacterial activity determination. Pure lactoferricin, prepared from pepsin-hydrolyzed Lf, was purified by standard chromatography techniques. This peptide was analyzed against a number of gram-positive and gram-negative bacteria before and after reduction of its disulfide bond or cleavage after its single methionine residue and was found to inhibit the growth of all the test bacteria at a concentration of 8 μM or less. Subfragments of lactoferricin were isolated from reduced and cleaved peptide by reverse-phase HPLC. Subfragment 1 (residues 1 to 10) was active against most of the test microorganisms at concentrations of 10 to 50 μM. Subfragment 2 (residues 11 to 26) was active against only a few microorganisms at concentrations up to 100 μM. These antibacterial studies indicate that the activity of lactoferricin is mainly, but not wholly, due to its N-terminal region.

Lactoferrin (Lf) is a bilobate iron-binding glycoprotein with a mass of approximately 80 kDa and is an antimicrobial component of milk and other exocrine secretions (27, 32). In serum, Lf is released by activated neutrophils during the inflammatory response, and there is in vivo evidence that it stimulates neutrophil accumulation and adhesion (33). Lf is also reported to be a regulator of myelopoiesis (13), although the mechanism is unclear (20). A strong iron-binding capability is implicated in its role as an effector of slow-cycle serum iron metabolism, where serum iron is bound by Lf and is subsequently taken up by receptors on macrophages and the liver for transfer to ferritin (10, 42, 43).

Lf is active against a wide selection of pathogenic bacteria (5, 14, 17, 38). Lf's strong iron-binding capacity is implicated in the antimicrobial mechanism, effectively withholding this essential element from otherwise proliferative microorganisms (17). There is also evidence indicating a direct killing effect, unrelated to iron binding, which is mediated by binding to the surfaces of susceptible organisms (4). The interaction between Lf and gram-negative bacteria releases significant levels of lipopolysaccharide from the outer membrane (19). This release, compromising outer membrane permeability, increases bacterial susceptibility to other antimicrobial factors such as lysozyme (18). Currently, there are no molecular studies which define Lf's antimicrobial mechanism, even though its three-dimensional structure is well characterized (3).

The stability of lactoferrin and the maintenance of its antibacterial character have been studied under a range of conditions. As a constituent of milk, Lf is subject to the enzymes and environment of the infant gut. Human Lf, absorbed via receptors in the gut lining, has been isolated structurally and functionally intact from the urine of infants fed human milk (22). A number of enzymes have since been used to test the functional integrity of Lf. The iron-binding capacity and antibacterial activity of bovine apolactoferrin are abolished by treatment with chymotrypsin or trypsin (11, 12). Treatments of bovine Lf with papain, actinase AS, protease P, protease A, or bioprase are also reported to eliminate both iron-binding and antimicrobial activities (38).

During studies to assess the heat stability of Lf, it was found that the protein was thermostable at acidic pH (1), but under more extreme conditions (120°C, pH 2) it produced a hydrolysate that retained antimicrobial activity independent of iron binding (35). Treatment of the protein with pepsin is also reported to yield a hydrolysate which, while unable to bind iron, remains antibacterially active (40). An active peptide, lactoferricin B (Lf-cin B), was subsequently isolated from the hydrolysate and identified as residues 17 to 41 of bovine Lf (7). Lf-cin B is highly basic, has a single disulfide bond and no iron-binding capacity, and is active against a wide range of microorganisms, including bacteria, yeast, and fungi (6, 8, 9, 44, 47). Peptides from the N-terminal region of Lf are also reported to have other biological activities. Two peptides (residues 39 to 42 and residues 20 to 37 of human Lf) have antithrombotic properties (26, 28, 46), while the first 14 N-terminal residues reportedly affect hepatic uptake of the protein (48). Fluorescent probes and peptide synthesis have identified a neutrophil-binding region on the N terminus (residues 4 to 52) of human Lf (25). These studies highlight the importance of Lf's N-terminal region to biological functions which do not directly involve iron binding.

Chymosin, like pepsin, is an aspartyl proteinase and demonstrates activity similar to that of pepsin at low pH. Previously, a study on the susceptibility of Lf to chymosin reported that native Lf is not hydrolyzed by the enzyme at pH 6.3 to 6.5 (37). In the present studies chymosin has been used under acidic

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conditions to generate several Lf-cin B-like peptides. We have used both membrane ion-exchange and standard chromatographic techniques to isolate antibacterially active lactoferricin from bovine Lf, and preliminary structural studies explored the antibacterial elements of the peptide. Purified lactoferricin was variously reduced at its single disulfide bond, cleaved at a centrally positioned peptide bond, or both reduced and cleaved to yield two subfragments. The antibacterial activities of these derivatives were compared to that of the intact peptide.

MATERIALS AND METHODS

Materials. Enterotoxigenic *Escherichia coli* L361 and *E. coli* O:9, isolated from piglets with neonatal or postweaning scours, were obtained from the Regional Veterinary Laboratory (Wagga Wagga, Australia). *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 bacteria were maintained on peptone yeast extract agar slants at 4°C and were subcultured every 2 months to ensure viability. Bovine Lf was purified from cheddar cheese whey by cation-exchange chromatography on S-Sepharose Fast Flow by the method of Law and Reiter (24). For reverse-phase high-pressure liquid chromatography (RP-HPLC) solvent A consisted of 0.1% trifluoroacetic acid (TFA; Auspep, Parkville, Australia), and solvent B was 0.09% TFA and 90% acetonitrile (Fisons Pty. Ltd., Homebush, Australia). Recombinant chymosin (Chymogen; EC 3.4.23.4) was a gift from R. Foo of Christian Hansen's Laboratory Pty. Ltd. (Bayswater, Australia). Pepsin A (EC 3.4.23.1) was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Generation of chymosin-hydrolyzed Lf and isolation of lactoferricins. Chymosin solution was dialyzed exhaustively against deionized water at 4°C to remove salts and preservatives. After dialysis the enzyme was lyophilized and stored at 4°C. Lf (5 g) was dissolved in 200 ml of water, and the solution was adjusted to pH 3.0 by dropwise addition of 1 M HCl. Recombinant chymosin (200 mg) was dissolved in the solution, which was incubated at 60°C for 1 h. The solution was cooled to 20°C, adjusted to pH 7.0 by dropwise addition of 1 M NaOH, and then centrifuged at 4°C for 15 min at 17,000 × *g*. The pellet was discarded. A Sartobind-S Minisart membrane ion-exchange (MIE) unit (Sartorius GmbH, Göttingen, Germany) was equilibrated with 10 ml of 10 mM sodium phosphate buffer (pH 7.0). Chymosin-hydrolyzed Lf (5 ml) was passed through the MIE unit, which was washed with 5 ml of 10 mM sodium phosphate buffer (pH 7.0). The MIE unit was then washed with 5 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 2 M NaCl and 10% acetonitrile. The procedure described above was repeated until 100 ml of chymosin-hydrolyzed Lf had been processed. The material eluting with the salt washes (retentate) was pooled and acidified to 0.1% TFA. The acidified material was loaded onto a preparative column (25 by 2.2 cm [inner diameter; i.d.]; Vydac C_{18}; 15 to 20 μm) which had been equilibrated at 5 ml/min with solvent A at 25°C. The column was washed with 100 ml of 20% solvent B, followed by a 40-min linear gradient to 35% solvent B. Fractions were collected every 1 min during the gradient, and elution was monitored by measuring the absorbances at 280 and 214 nm. Purified peptides were lyophilized and were stored at −20°C.

Generation of pepsin-hydrolyzed Lf and isolation of lactoferricin. The generation of pepsin hydrolysate of Lf followed closely the methods of Tomita et al. (40). Briefly, 10 g of Lf was dissolved in water and was adjusted to pH 2.5 by dropwise addition of 1 M HCl. Pepsin (300 mg) was dissolved in the solution, which was incubated at 37°C for 4 h. The reaction was terminated by incubation of the solution at 80°C for 15 min. The solution was cooled to 20°C, adjusted to pH 7.0 by dropwise addition of 1 M NaOH, and then centrifuged at 4°C for 15 min at 17,000 × *g*. The pellet was discarded. A column (25 by 2.2 cm [i.d.]) of S-Sepharose Fast Flow resin was equilibrated at 25°C and at a flow rate of 2 ml/min with 50 mM sodium phosphate buffer (pH 7.0) containing 10% acetonitrile. Pepsin-hydrolyzed Lf was loaded onto the column, and the column was washed with 50 mM sodium phosphate buffer (pH 7.0) containing 10% acetonitrile. The column was then washed with 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 10% acetonitrile, followed by a final wash with 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl and 10% acetonitrile. Bulk fractions were characterized by analytical RP-HPLC as follows. A small aliquot (10 μl) was acidified to 0.1% TFA and loaded onto an analytical column (25 by 0.46 cm [i.d.]; Vydac C_{18}, 5 μm) equilibrated with 20% solvent B at 1 ml/min and 25°C; the column was then subjected to a 40-min linear gradient from 20% solvent B to 50% solvent B. The material identified by analytical HPLC as containing lactoferricin was acidified to 0.1% TFA and was loaded onto a preparative column (25 by 2.2 cm [i.d.]; Vydac C_{18}) which had been equilibrated at 5 ml/min with solvent A at 25°C. The column was washed with 100 ml of 20% solvent B, followed by a 40-min linear gradient to 35% solvent B. Fractions were collected every 1 min during the gradient, and elution was monitored by measuring the absorbances at 280 and 214 nm. Purified peptide was lyophilized and stored at −20°C.

Antibacterial assay. The antibacterial assay used was based on the method of Nonnecke and Smith (30). Incubations for all organisms except *P. fluorescens* were done at 37°C; *P. fluorescens* was incubated at 30°C. Working inocula of bacteria were prepared by subculture of isolates from agar slants into Todd-Hewitt broth and incubation for 16 h. After this time 0.5 ml of the culture was inoculated into 10 ml of fresh Todd-Hewitt broth, and the mixture was incubated for a further 6 h. The culture was then diluted in 0.1% peptone to approximately 10^4 CFU/ml and was used for inoculation of the microassay. Assays were performed in sterile 96-well tissue culture plates (Disposable Products, Technology Park, Australia). The growth medium contained 1% peptone, 0.05% yeast extract, and 1% glucose (PYG medium; pH 6.8). Solutions of test peptide were prepared in filter-sterilized water. To each well was added 190 μl of PYG medium, 50 μl of peptide solution, and 10 μl of bacterial inoculum. Growth controls contained sterile water in place of peptide solutions, and blanks contained sterile water in place of both peptide solutions and bacterial inocula. The plate was covered with a sterile lid and was incubated for 17 h. After incubation each well was scanned for the absorbance at 620 nm. Antibacterial activity was expressed as the MIC of peptide required to give no increase in absorbance at 620 nm following incubation. All analyses were performed in duplicate three times.

Chemical modification of lactoferricin. A method based on that of Allan (2) was used to cleave the peptide with cyanogen bromide (CNBr). The peptide was dissolved in 70% acetic acid with CNBr at a molar ratio of 1:100 (peptide:CNBr). The solution was flushed with nitrogen gas and sealed and was then incubated overnight at 25°C with constant stirring. After the reaction, the solution was diluted eightfold in water and lyophilized. It was then redissolved in water and lyophilized again. The dry product was stored at −20°C. Reduction of lactoferricin was carried out by dissolving purified peptide in 250 mM β-mercaptoethanol in 50 mM sodium phosphate buffer (pH 8.0) and heating for 5 min in a boiling water bath. After reduction, the solution was acidified to 0.1% TFA and was subjected to RP-HPLC to remove excess β-mercaptoethanol and the di-β-hydroxyethyl disulfide reaction product. The procedure was as follows. The acidified sample was loaded onto a preparative RP-HPLC column (25 by 2.2 cm [i.d.]; Vydac C_{18}) which had been equilibrated at 5 ml/min with solvent A at 25°C. The column was washed with 100 ml of 20% B, followed by a 40-min linear gradient to 35% solvent B. Fractions were collected every 1 min during the gradient, and elution was monitored by measuring the absorbances at 280 and 214 nm. Aliquots (50 μl) of each fraction were added to 950 μl of solvent A, and the solution was analyzed by analytical RP-HPLC. Fractions containing the reduced peptide were pooled, lyophilized, and stored at −20°C.

RP-HPLC isolation of CNBr fragments of lactoferricin. CNBr-cleaved peptide was dissolved in 250 mM β-mercaptoethanol in the presence of 50 mM sodium phosphate buffer (pH 8.0), and the solution was heated for 5 min in a boiling water bath. The sample was acidified to 0.1% TFA and was purified by preparative RP-HPLC as described above for reduced lactoferricin. Purified peptides were lyophilized and stored at −20°C.

MS. The mass of each purified peptide was assessed by mass spectrometry (MS) by using a PE-SCIEX AP*111* triple quadrupole mass spectrometer fitted with an Ion Spray ion source (PE-SCIEX Instruments, Toronto, Ontario, Canada). Sample (5 μl) was injected into the solvent delivery line (solvent mixture, 45% acetonitrile in 0.05% TFA) of the mass spectrometer operated in the positive ion mode (Ion Spray voltage, +5 kV). Standard scan conditions were m/z 300 to 2,400 in 5 s by using a step of 0.5 atomic mass units. Spectra were collected by using multichannel averaging at an orifice potential of 70 V. Molecular masses were calculated by using HyperMass software, and raw data were analyzed by MacBioSpec, version 1.0.1, and MacSpec, version 3.2, software (PE-SCIEX Instruments).

N-terminal sequencing. N-terminal sequencing of pure peptides was performed by Edman degradation with an Applied Biosystems 470A protein sequencer with an on-line model 120A PTH Analyzer (Applied Biosystems, Foster City, Calif.). Typically, five cycles were run. Sequencing results were combined with mass spectral analysis and the full amino acid sequence of bovine Lf (34) in order to identify the full sequence of each fragment.

RESULTS

Purification and characterization of chymosin-generated lactoferricins. In an attempt to generate and isolate antimicrobial peptides, Lf was hydrolyzed with chymosin at low pH, and the hydrolysate was passed through a cation-exchange filter unit. Analytical and preparative loads of Lf hydrolysate were compared to determine if active peptides might be purified by displacement chromatography. MIE of analytical (1 ml) and preparative (5 ml) loads resulted in retentates with markedly different RP-HPLC profiles (Fig. 1). The analytical retentate revealed by RP-HPLC a six-peak profile which was reduced to three in the preparative retentate. Peptides which eluted with 2 M NaCl from the MIE unit under preparative conditions

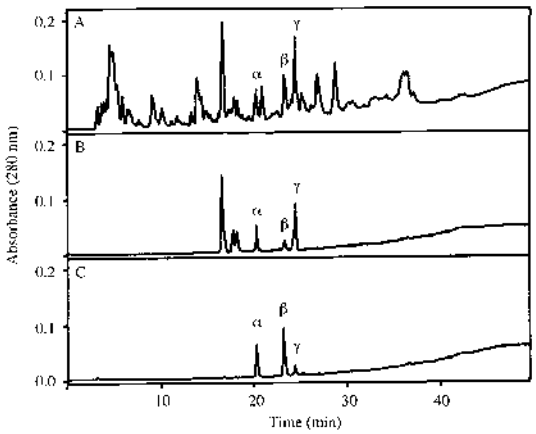

FIG. 1. Analytical RP-HPLC of chymosin-hydrolyzed Lf and MIE retentate fractions. Samples were brought to 0.1% TFA, and 10 μl was injected onto a Vydac C_{18} column (25 by 0.46 cm [i.d.]; solvent A, 0.1% TFA; solvent B, 90% acetonitrile in 0.09% TFA; equilibration was in 20% solvent B for 5 min; gradient, 20% solvent B to 50% solvent B over 40 min; flow rate, 1 ml/min). A Sartobind-S Minisart MIE unit was used to fractionate chymosin-hydrolyzed Lf by using analytical (1 ml) or preparative (5 ml) loads of hydrolysate. (A) Chymosin-hydrolyzed Lf. (B) Analytical MIE retentate. (C) Preparative MIE retentate. Peak α was characterized as peptides 1A and 1B, peak β represents peptide 2, and peak γ represents peptide 3.

were further fractionated by RP-HPLC. Isolation and characterization of the earliest of three RP-HPLC peaks (α) revealed the copurification of two peptides with identical N termini (FKCRR) and RP-HPLC retention times. Mass spectral analysis confirmed the presence of two species with molecular masses of 3,123 Da (peptide 1A) and 3,194 Da (peptide 1B). The two peptides appeared to be produced in a ratio of approximately 1:1 (mass spectral data not shown). The next eluting peak (β) was a single peptide, also with an N terminus of FKCRR, but having a molecular mass of 3,308 Da (peptide 2). The last peak (γ) was also due to a single species, consisting of two peptides joined by a disulfide bond (a heterodimer) and having a molecular mass of 5,850 Da (peptide 3). The two N termini of the heterodimer were determined by sequencing to be FKCRR and APRKN. The full sequences of the peptides are presented in Fig. 2. Antibacterial assays were performed to determine the activities of the peptides. The peptide mixture retained by the MIE unit had an MIC of 20 μg/ml for *E. coli* under hypotonic conditions. The RP-HPLC-copurified peptides, peptide 1A and peptide 1B, had MICs of 12.5 μg/ml (4 μM). The other two RP-HPLC-purified peptides (peptides 2 and 3) also had MICs of 4 μM (Table 1).

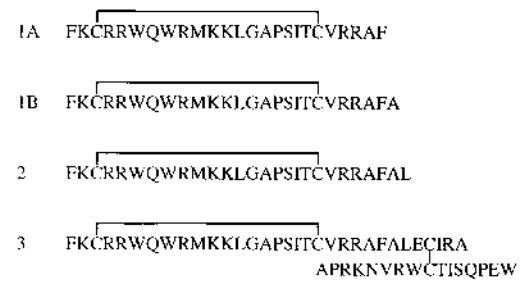

FIG. 2. Sequences of antibacterial peptides isolated from chymosin-hydrolyzed Lf. Primary structures were determined by MS, N-terminal Edman sequencing, and reference to the known sequence of bovine Lf (34). Peptides 1A and 1B copurify during RP-HPLC. Peptide 1A is identified as lactoferricin B (7). Lines connecting cysteine residues indicate disulfide bonds.

TABLE 1. Antibacterial activities of chymosin-hydrolyzed Lf and peptides against *E. coli* L361

Lf or peptide	MIC[a]	
	μg/ml	μM
Native Lf[b]	1,000	12.5
Chymosin-hydrolyzed Lf[b]	400	
Membrane ion-exchange retentate[c]	20	
Peptides 1A and 1B[d]	12.5	4
Peptide 2[d]	13.2	4
Peptide 3[d]	23.4	4

[a] Defined as the minimum concentration required to inhibit bacterial growth, measured by monitoring the absorbance at 620 nm.
[b] Concentrations of 0, 200, 400, 600, 800, and 1,000 μg/ml were used.
[c] Retentate concentrations of 0, 10, 20, 30, 40, and 50 μg/ml were used.
[d] Peptide concentrations of 0, 2, 4, 6, 8, and 10 μM were used. For peptides 1A and 1B, concentration was based on the total weight of material containing both components and using an average value of the molecular mass (their molecular masses differ by 2%).

Purification and characterization of pepsin-generated lactoferricin. To generate the antimicrobial peptide lactoferricin, Lf was enzymatically digested with pepsin. Partial purification of lactoferricin from the hydrolysate, achieved by cation-exchange chromatography, is illustrated in Fig. 3. Final purification of the peptide was effected after RP-HPLC; homogeneity was determined by HPLC (Fig. 4) and mass spectrometry (data not shown). The average yield of lactoferricin by this method from a theoretical maximum was 50% (200 mg from 10 g of Lf). The peptide had a molecular mass of 3,194 Da and an N-terminal sequence of FKCRR, revealing it to be comprised of residues 17 to 42 in bovine Lf (FKCRRWQWRMKKLGA PSITCVRRAFA), with a disulfide bond between Cys_3 and Cys_{20} of the peptide (Fig. 5). The calculated monoisotopic molecular mass of the oxidized form of the peptide is 3,193.7 Da. The antibacterial activity of purified lactoferricin was determined against a number of bacteria (Table 2). The peptide's apparent MIC ranged from 2 to 8 μM.

Reduction and cleavage of lactoferricin. To determine whether disruption of its cyclic structure would have an effect on the antimicrobial activity of lactoferricin, the peptide was either reduced in the presence of β-mercaptoethanol or

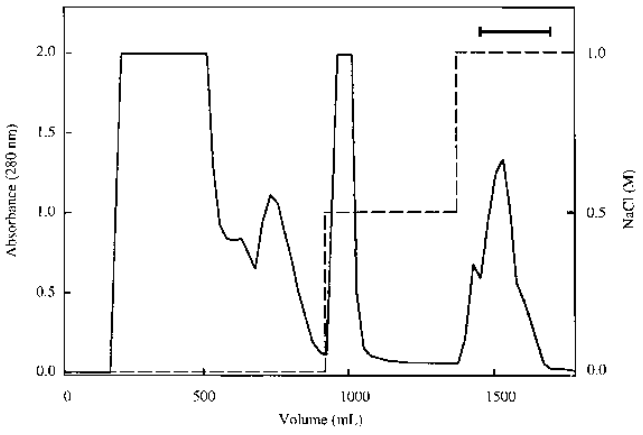

FIG. 3. S-Sepharose Fast Flow chromatography of pepsin-hydrolyzed Lf. The supernatant (pH 7.0) from 10 g of pepsin-hydrolyzed Lf was loaded onto a column (25 by 2.5 cm [i.d.]) and eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 10% acetonitrile at a flow rate of 2 ml/min. A two-step NaCl gradient (0.5 and 1 M) was used to fractionate the hydrolysate (– – – –). The fraction containing lactoferricin is indicated by the bar at the top right.

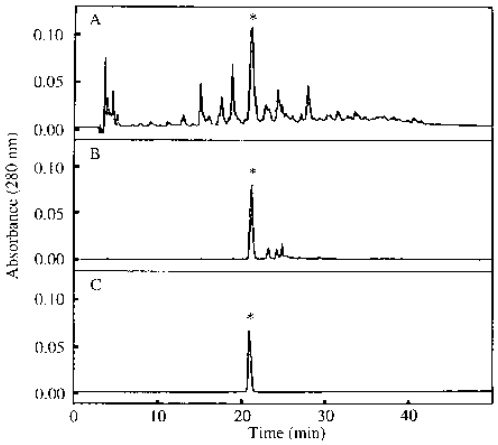

FIG. 4. Analytical RP-HPLC of ion-exchange and preparative HPLC fractions of pepsin-hydrolyzed Lf that contain lactoferricin. Samples were brought to 0.1% TFA and 10 μl was injected onto a Vydac C_{18} column (25 by 0.46 cm [i.d.]; solvent A, 0.1% TFA; solvent B, 90% acetonitrile in 0.09% TFA; equilibration was in 20% solvent B for 5 min; gradient, 20% solvent B to 50% solvent B over 40 min; flow rate, 1 ml/min). (A) Pepsin-hydrolyzed Lf. (B) Ion-exchange fraction containing lactoferricin. (C) RP-HPLC-purified lactoferricin. The elution position of lactoferricin is indicated with asterisks.

cleaved with CNBr. Reduction of the peptide's disulfide bond resulted in a molecular mass increase of 2, to 3,196 Da. Analysis of this reduced peptide by RP-HPLC showed an increase in the retention time, from 20.7 to 22.3 min. Antibacterial assays comparing the oxidized (untreated) and reduced forms of lactoferricin were carried out against a number of bacteria. The MIC of oxidized lactoferricin varied from 2 to 8 μM (Table 2). Reduction of the disulfide bond did not greatly alter the peptide's antibacterial activity (Table 2). Cleavage of oxidized lactoferricin by CNBr in the presence of acetic acid resulted in a mass of 3,164 Da, a decrease of 30 Da, consistent with the formation of a homoserine lactone at Met_{10}. The retention time on RP-HPLC decreased to 19.5 min from 20.7 min. The same reaction carried out in the presence of formic acid resulted in the formation of two products with masses of 3,164 and 3,192 Da. Analysis by MS-MS suggested that the second product of CNBr-formic acid cleavage was due to the reversible esterification of a serine residue (data not shown). Antibacterial assays with the CNBr-acetic acid-cleaved peptide

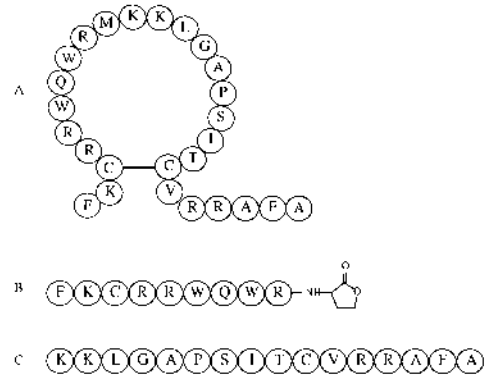

FIG. 5. The primary sequence of lactoferricin and CNBr-derived subfragments. The peptides were identified by N-terminal sequencing and MS. (A) Lactoferricin, differing from lactoferricin B (7) by the presence of a C-terminal alanine residue. (B) Subfragment 1, including the C-terminal homoserine lactone. (C) Subfragment 2.

TABLE 2. Antibacterial activities of lactoferricin and its derivatives

Bacterium	MIC[a] (μM) of lactoferricin[b]		
	Untreated	Reduced	CNBr-cleaved
Listeria monocytogenes	2	4	2
Escherichia coli L361	4	4	4
Salmonella Salford	4	4	4
Staphylococcus aureus	6	6	6
Escherichia coli O:9	6	8	6
Bacillus cereus	6	8	6
Pseudomonas fluorescens	8	6	8

[a] Defined as the minimum concentration required to inhibit bacterial growth, measured by monitoring the absorbance at 620 nm.
[b] Peptide concentrations of 0, 2, 4, 6, 8, and 10 μM (0, 6.3, 12.5, 18.8, 25, and 31.3 μg/ml, respectively) were used.

demonstrated that this treatment had no measurable effect on the activity of lactoferricin against a number of bacteria (Table 2).

Fragmentation of lactoferricin. To determine whether the antibacterial activity of lactoferricin resides in one region of the peptide or another, CNBr-cleaved lactoferricin was reduced with β-mercaptoethanol, and subsequently, purified fragments were assayed for their antibacterial activities. Subfragment 1 had a molecular mass of 1,448 Da and an N-terminal sequence of FKCRR, consistent with the predicted sequence FKCRRWQWRm′, which represents the N-terminal subfragment of the peptide (Fig. 5). The antibacterial activity of this subfragment was not as potent as that of intact lactoferricin, having an MIC of 10 to 50 μM for most of the bacteria tested (Table 3). *Salmonella* Salford and *S. aureus* remained unaffected by concentrations up to 50 μM. Subfragment 2 had a molecular mass of 1,718 Da and an N-terminal sequence of KKLGA, consistent with the predicted sequence of KKLGA PSITCVRRAFA, the C-terminal subfragment of the peptide (Fig. 5). Subfragment 2 had weak antibacterial activity against *L. monocytogenes* and both *E. coli* isolates, being effective in the range of 40 to 60 μM. All other isolates remained unaffected at concentrations up to 100 μM (Table 3).

DISCUSSION

These studies indicate that treatment of Lf with recombinant chymosin at acidic pH can be used to generate an antibacterial hydrolysate. Furthermore, by using an adaptation of the displacement chromatography technique (21), a single-step ion-exchange fractionation can yield a mixture of peptides identical

TABLE 3. Antibacterial activities of fragments of lactoferricin

Bacterium	MIC[a] (μM [μg/ml])	
	Subfragment 1	Subfragment 2
Listeria monocytogenes	10 (14.5)[b]	40 (68.7)[c]
Escherichia coli L361	10 (14.5)[b]	50 (85.9)[c]
Escherichia coli O:9	15 (21.7)[d]	60 (103.1)[e]
Bacillus cereus	20 (29.0)[d]	>100 (>171.8)[e]
Pseudomonas fluorescens	50 (72.4)[c]	>100 (>171.8)[e]
Salmonella Salford	>50 (>72.4)[c]	>100 (>171.8)[e]
Staphylococcus aureus	>50 (>72.4)[c]	>100 (>171.8)[e]

[a] Defined as the minimum concentration required to inhibit bacterial growth, measured by monitoring the absorbance at 620 nm.
[b] Peptide concentrations of 0, 2, 4, 6, 8, and 10 μM were used.
[c] Peptide concentrations of 0, 10, 20, 30, 40, and 50 μM were used.
[d] Peptide concentrations of 0, 5, 10, 15, 20, and 25 μM were used.
[e] Peptide concentrations of 0, 20, 40, 60, 80, and 100 μM were used.

or closely homologous to the antibacterial peptide Lf-cin B characterized by Bellamy et al. (7). The antibacterial activities of the peptides obtained by these methods are comparable to that of Lf-cin B. The activity of the heterodimer (peptide 3) was unaffected by structural additions at its C terminus, suggesting that the region of activity for lactoferricin peptides lies within their N-terminal sequences. Other studies undertaken in this laboratory have observed that chymosin digestion of apolactoferrin at slightly higher pH yields two large antibacterial N-terminal fragments (Lf_{1-106} and Lf_{1-92}). The iron-binding residues of Lf are well characterized (3), and it is speculated that the fragments do not bind iron because they lack all but one of the necessary iron-binding residues. Initial tests suggest that the antibacterial activity of these larger fragments (MIC = 160 μg/ml [13 μM]) closely resembles that of native Lf (MIC = 1 mg/ml [12.5 μM]).

From studies performed with chymosin and Lf at physiological pH (37), it is unlikely that during the production of cheese any antimicrobial peptides are produced by chymosin hydrolysis. Essentially all of the proteolysis that occurs during coagulation is effected at a single site on κ-casein (15). However, the acids and enzymes of the mammalian gut that ingested Lf encounters may provide an excellent in vivo environment for the production of lactoferricins. In support of this, Tomita (39) has isolated and characterized Lf-cin B excreted by Lf-fed rats, suggesting a biological role for Lf as a precursor for antimicrobial lactoferricin peptides. The large-scale production of Lf from cheese whey (16) or by fermentation (45) is well developed. Because Chymogen is a cheaper and readily obtainable alternative to other aspartic proteases, it appears feasible for dairy technologists to treat Lf with chymosin under acidic conditions and produce large quantities of antibacterial hydrolysate. Technology developed for Lf purification could also be used to isolate and purify antibacterially active peptides in considerable quantities.

The isolation procedure used to obtain pepsin-generated lactoferricin relied primarily on ion-exchange chromatography to achieve a high yield and purity. The difference between the lactoferricin isolated in this study (26 residues) and Lf-cin B (25 residues) may be accounted for by the methods used to characterize the peptides; Lf-cin B was characterized solely by N-terminal sequencing (7), while the full sequence reported here was determined by a combination of N-terminal sequencing and MS. It appears possible that if Lf-cin B were characterized more extensively, its sequence may well have been found to include a C-terminal alanine.

Each of the derivatives and subfragments of lactoferricin retained at least some antibacterial activity. Against a range of gram-positive and gram-negative bacteria, the activity of lactoferricin did not vary appreciably, because all isolates were inhibited by peptide concentrations within the range of 2 to 8 μM. The activity of reduced lactoferricin confirms the work of Bellamy et al. (7), where a synthesized analog of Lf-cin B with covalently blocked cysteine (acetamidomethyl-cysteine) residues matched the activity of pepsin-generated Lf-cin B. This is in contrast to the findings made with bactenecin, a bovine neutrophil dodecapeptide with an internal disulfide bond, which lost all antibacterial activity on breaking its cyclic structure by replacement of disulfide-forming cysteines with alanines (41). Breaking the cyclic structure of lactoferricin by cleavage of the peptide at a point distal to its intact disulfide bridge also resulted in no change to its antibacterial activity. This suggests that neither a cyclic nor a linear structure for the peptide is particularly critical for its effectiveness as an antimicrobial agent. In a similar study, the lipopolysaccharide-binding region of the 18-kDa cationic antibacterial protein (CAP18) of rabbit neutrophils was synthesized as a 37-residue peptide ($CAP18_{106-142}$) (23). The sequence was found to be antibacterial, and shorter peptides of $CAP18_{106-142}$ were synthesized to determine the antibacterial domain. However, two truncated peptides based on the 37-residue sequence, equivalent to $CAP18_{106-114}$ and $CAP18_{117-142}$, respectively, had no activity either individually or in combination. This suggested that the complete antimicrobial sequence must have specific structural requirements not met by the shorter analogs (23).

As for $CAP18_{106-142}$, the antibacterial activity of lactoferricin, by these findings, could not be localized to a single region of the peptide. However, unlike the shorter analogs of $CAP18_{106-142}$, both fragments of lactoferricin retained antibacterial activity. This activity can be primarily attributed to the first 10 N-terminal residues and partially to its remainder. At physiological pH subfragment 1 has a higher positive charge (five positive charges) than subfragment 2 (four positive charges), and this charge difference may partially account for the difference in antibacterial activity. It is doubtful, however, that a high basic charge is solely responsible for the activity recorded for these peptides. Experiments which combined equimolar concentrations of subfragments 1 and 2 did not return the activities obtained with CNBr-cleaved and oxidized lactoferricin, suggesting that each subfragment contains an element that must be brought into proximity with the other to achieve the potent activity displayed by intact lactoferricin. Determination of the absolute nature of the peptide's antibacterial character would involve structural studies and further chemical modification, initially directed at its positively charged residues.

Current opinion on the mechanism of action of basic antibacterial peptides is focused on their interaction with the negatively charged elements in the membranes of susceptible bacteria (29). These elements are lipopolysaccharide (LPS) in gram-negative bacteria and lipotechoic acid in gram-positive bacteria. Polymyxin B, a polycationic cyclic decapeptide with an attached fatty acid is proposed to interact with LPS phosphate groups of the gram-negative outer membrane by ionic attraction. It is reported to follow this by inserting its fatty acid into the bilayer and distorting the lipid arrangement (36). Subsequent work determined that while removal of the terminal fatty acyl diaminobutyric acid of polymyxin B effectively abrogated its own antibacterial character, it was still capable of sensitizing bacterial membranes to several antibiotics (31). It seems likely that the nonspecific antibacterial activity of lactoferricin and its subfragments is due to a similar interaction with structures of a generalized nature on the surface of susceptible microorganisms (e.g., LPS of gram-negative bacteria or lipotechoic acid of gram-positive species) and that this activity involves a compromise of the membrane permeability barrier.

ACKNOWLEDGMENTS

The assistance of A. Jones and M. Loughnan of the Centre for Drug Design and Development (University of Queensland, Brisbane, Australia) and the technical help of T. Beattie are gratefully acknowledged. Bovine Lf was kindly provided by G. Smithers of CSIRO Division of Food Science and Technology (Highett, Australia).

This research was supported by a grant from the Australian Dairy Research and Development Corporation.

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