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Purification, Composition, and Activity of Two Bactenecins, Antibacterial Peptides of Bovine Neutrophils

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Extracts of granules of bovine neutrophils are known to exhibit a marked antibacterial activity in vitro. By a simple, two-step chromatographic procedure, we have resolved two peptide components of the antibacterial system. They were named Bac-5 and Bac-7 from the general term bactenecin and had molecular masses of about 5 and 7 kilodaltons, respectively. Over 45 and 20% of the amino acid residues in the two bactenecins are proline and arginine, respectively. The remaining amino acids are mainly hydrophobic (isoleucine, leucine, and phenylalanine). Both Bac-5 and Bac-7 efficiently kill *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae*. They also arrest the growth of *Enterobacter cloacae* (MICs, 25 to 200 μg/ml) but not of *Proteus vulgaris*, *Staphylococcus aureus*, and *Streptococcus agalactiae* (MIC, >200 μg/ml). Finally, Bac-7 but not Bac-5 has MICs of ≤200 μg/ml for *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. From the comparison between the efficient bactericidal concentrations in vitro and the estimated content of bactenecins in neutrophils (125 ng of Bac-5 and Bac-7 each per 10^6 cells), it is reasonable to conclude that the two cationic peptides may exert a major role in host defense against at least some microorganisms.

The most prominent population of granules in neutrophils of ruminants is larger and more dense than the well-known azurophil and specific granules and contains neither the usual lysosomal hydrolases nor neutral proteases (1, 5). Instead, it contains a unique group of strongly cationic polypeptides, which are likely to be responsible for the oxygen-independent antimicrobial activity of the cell. In fact, among the three granule classes, the large granules are the only ones to display an antibacterial activity in vitro. Furthermore, upon phagocytosis, both the antibacterial activity and the cationic polypeptides are released into the extracellular medium (5).

Working with bovine neutrophils, we have shown in previous investigations that, once extracted from the granules, these polypeptides can be resolved into distinct pools with various levels of effectiveness against an array of gram-negative and gram-positive bacteria (6, 7, 10). Independently of their composition and activity, these cationic polypeptides have been collectively called bactenecins, from the Latin words bacterium and necare (to kill). The first bactenecin we have purified and characterized is a dodecapeptide, maintained in a cyclic structure by a disulfide bond, which is very active against both *Escherichia coli* and *Staphylococcus aureus* (8). Here, we describe the purification and properties of two additional bactenecins, which can suppress the growth of gram-negative bacteria and are characterized by a high content in proline and arginine.

MATERIALS AND METHODS

Chemicals. High-pressure liquid chromatography-grade acetonitrile was purchased from Riedel-de-Haen AG (Seelze, Federal Republic of Germany), trifluoroacetic acid (TFA) was from Janssen Chimica (Beerse, Belgium), acrylamide and other electrophoresis reagents were from Bio-Rad Laboratories (Richmond, Calif.), and water was Milli Q grade from Millipore Corp. (Bedford, Mass.).

Preparation of the granular extract. Bovine neutrophils (about 1.5×10^{10} per batch) were isolated from freshly collected blood, and the total granular population was separated from postnuclear supernatants as previously described (6). The granule pellets of several preparations were pooled and then extracted for 2 h in an ice bath with 0.2 M sodium acetate buffer, pH 4.0, containing 5 mM EDTA. The insoluble material was sedimented at 30,000 × *g* for 30 min and reextracted as described above. The two extracts were combined and dialyzed in a Spectrapore 6 membrane tubing (nominal molecular weight cutoff, 1,000; Spectrum Medical Ind., Los Angeles, Calif.) against water acidified at ca. pH 3 with HCl. The dialyzate was then lyophilized and redissolved in 10 mM sodium phosphate buffer, pH 7, containing 0.2 M NaCl and centrifuged for 40 min at 30,000 × *g* to remove any undissolved material.

Purification of the antibacterial peptides. (i) Ion-exchange chromatography. The granular extract was applied to a cation-exchange carboxymethyl-cellulose column (1.5 by 15 cm; CM 52; Whatman Inc., Maidstone, Kent, United Kingdom), equilibrated with 0.2 M NaCl in 10 mM sodium phosphate, pH 7. After extensive washing with the same solution to remove unbound proteins, the content of NaCl in the elution medium was increased to 0.3 M. This change in ionic strength caused the elution of three peaks whose fractions were combined into corresponding pools, named (in order of appearance) IIa, IIb, and IIc.

(ii) RPC. Pools IIb and IIc were dialyzed against HCl-acidified water (ca. pH 3) and then vacuum-dried. The lyophilysate thus obtained was redissolved in 0.1% TFA and subjected either to preparative reversed-phase chromatography (RPC) with a Pep-RPC HR 10/10 column or to analytical RPC with a Pep-RPC HR 5/5 column of the fast protein liquid chromatography system (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Elution of the bound material, monitored at 214 nm, was performed with a 0 to 100% acetonitrile gradient in 0.1% TFA at flow rates of 2.0 and 0.7 ml/min for the preparative and the analytical chromatography, respectively. The fractions of each resolved peak were

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pooled and lyophilized after removal of acetonitrile by flushing with nitrogen.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions by forming a 10 to 30% linear gradient with 10% acrylamide–0.27% *N,N'*-methylenebisacrylamide and 30% acrylamide–1.6% *N,N'*-methylenebisacrylamide. Acid urea-PAGE was conducted on slabs generated from 20% acrylamide–0.5% *N,N'*-methylenebisacrylamide. The gel slabs were 7 cm long and were stained with Coomassie brilliant blue R.

Amino acid analysis. After dried portions of the two purified peptides (ca. 10 nmol) were hydrolyzed for 22, 48, or 72 h at 110°C in evacuated sealed tubes with 0.3 ml of 6 N HCl, the amino acid content of the hydrolysates was analyzed with an automatic amino acid analyzer (Carlo Erba, Milan, Italy).

Protein determination. Protein was measured by the method of Waddel, as modified by Romeo et al. (9), with bovine serum albumin as the standard.

Microorganisms and evaluation of MIC. To evaluate the antibacterial activity of the two bactenecins, the following strains were used: *E. coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Enterobacter cloacae* ATCC 13047, *Salmonella typhimurium* ATCC 14028 and LT2, *Pseudomonas aeruginosa* ATCC 7700, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus agalactiae* ATCC 13813.

The microorganisms were grown either in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) or in Iso-Sensitest broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). After cultivation for 16 to 18 h at 37°C, the bacteria were harvested, and their density was determined by measuring A_{600} and referring to previously determined standards. MICs of the bactenecins were determined by a microdilution susceptibility test in sterile 96-well microdilution plates (Sterilin, Teddington, Middlesex, United Kingdom). Microorganisms (1×10^4 to 2×10^4 CFU/50 μl) were pipetted into the wells, which contained serial 1:1 dilutions (50 μl) of the peptides. The highest peptide concentration tested was 200 μg/ml. The plates were then incubated overnight at 37°C for MIC evaluation. Controls were run by replacing the peptide solution in TFA with 0.1% TFA alone (maximal final concentration, 0.005%).

Evaluation of bactericidal activity. The bactericidal activity of the purified neutrophil peptides was evaluated by using *E. coli*, *Salmonella typhimurium* ATCC 14028, and *K. pneumoniae* as test organisms. The bacteria were generally used in mid-logarithmic phase, with the exception of *E. coli*, which was also tested in stationary cultures. In the former case, 1 ml of overnight culture in liquid nutrient broth (Difco) was added to 50 ml of fresh nutrient broth and subcultured with stirring for 3 to 4 h at 37°C. The bacteria were subsequently sedimented at 1,000 × *g* for 10 min and washed by centrifugation with 10 mM sodium phosphate–100 mM NaCl, pH 7.4 (buffer A). Stationary cultures of *E. coli* were obtained by growing the organism overnight at 37°C on solid nutrient broth and then harvesting colonies in buffer A. In both cases, the density of the bacterial suspensions was determined by measuring their A_{600} as described above.

Incubation mixtures for the bactericidal assays contained 5×10^6 to 10×10^6 CFU of the mid-log-phase bacteria per ml or 0.7×10^6 to 1.5×10^6 CFU of the stationary-phase organisms per ml and 5 to 50 μg of the bactenecins per ml in a final volume of 200 μl of buffer A. Controls lacked the polypeptides but contained TFA at a maximal final concentration of 0.005%. After incubation at 37°C for the appropriate time, samples were serially 10-fold diluted with buffered saline (pH 7.4), plated in duplicate on nutrient agar, and incubated for 16 to 18 h to allow colony counts.

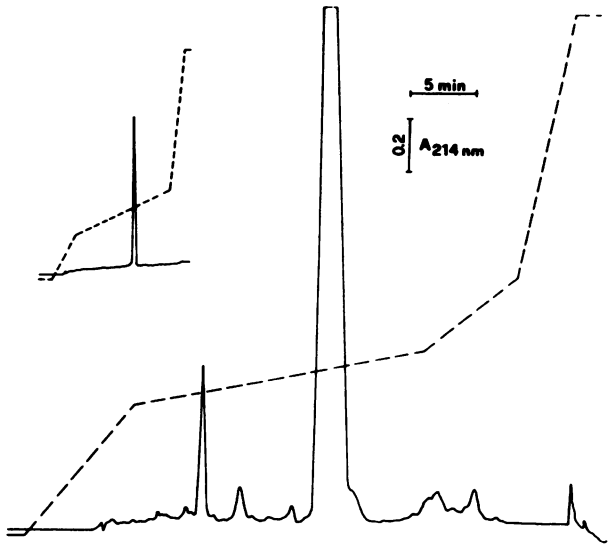

FIG. 1. RP-high-pressure liquid chromatography of protein pool IIb. Lyophilized protein (800 μg) of pool IIb was dissolved in 0.1% TFA and loaded on a preparative Pep-RPC HR 10/10 column of the fast protein liquid chromatography system. – – –, Shape of the 0 to 100% acetonitrile gradient used to elute the column. The major peak corresponds to Bac-5. Inset, Homogeneity of the purified bactenecin as determined with an analytical Pep-RPC HR 5/5 column.

RESULTS

Purification of the bactenecins. Batchwise processing of 3.2×10^{11} cells yielded about 2.6 g of protein in the acidic granule extract. This material, subjected to cation-exchange chromatography followed by preparative RPC, generated about 40 mg of each bactenecin.

Three peaks, named (in order of appearance) IIa, IIb, and IIc, were obtained by eluting the carboxymethyl-cellulose column with 0.3 M NaCl in 10 mM sodium phosphate, pH 7.0. Pools IIb and IIc exhibited considerable antibacterial activity against *E. coli* and contained a limited number of polypeptides.

To purify the antibacterial components, samples of each protein pool were loaded on a Pep-RPC column of the fast protein liquid chromatography system. Elution with 0 to 100% acetonitrile gradients of appropriate shape in 0.1% TFA led to resolution of the components present in each pool. Elution profiles from the RPC column of pools IIb and IIc, monitored at 214 nm (as many components did not adsorb at 280 nm), are shown in Fig. 1 and 2. The major peak of each profile contained the active bactenecin. Evidence for homogeneity of the purified peptides was provided by analytical RPC (see insets in Fig. 1 and 2), SDS-PAGE (Fig. 3, lanes b and c) and acid urea-PAGE (Fig. 3, lanes e and f). In SDS-PAGE, the antibacterial peptide purified from pool IIb had an apparent molecular weight of 4,300 (Fig. 3, lane c), whereas the bactenecin purified from pool IIc had an apparent molecular weight of 7,500 (Fig. 3, lane b). Analyses of the amino acid sequences of these two peptides (unpublished data) showed that the two bactenecins have molecular

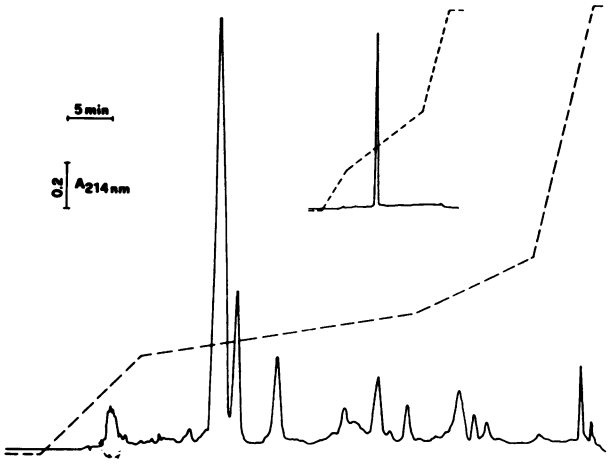

FIG. 2. RP-high-pressure liquid chromatography of protein pool IIc. Lyophilized protein (400 μg) of pool IIc was processed as described in the legend to Fig. 1. The major peak corresponds to Bac-7. Inset, Homogeneity of the purified bactenecin as described in the legend to Fig. 1.

weights of about 5,000 and 7,000, respectively. They were thus called Bac-5 and Bac-7.

Assuming a final yield of 100% for the purification procedure, the two bactenecins each represented about 1.5% of the protein of the granule extract or about 0.15% of the total cell protein.

Characterization of the bactenecins. (i) Amino acid analysis. Amino acid analysis (Table 1) indicated that Bac-5 and Bac-7 contain only a limited number of amino acid types and are both characterized by a high content of proline (>45%) and arginine (>20%) residues, the latter conferring a highly cationic nature on these polypeptides. The remaining amino acid residues (leucine, isoleucine and phenylalanine) are mainly hydrophobic.

(ii) Antibacterial activity. The antimicrobial properties of the two bactenecins were investigated with an array of

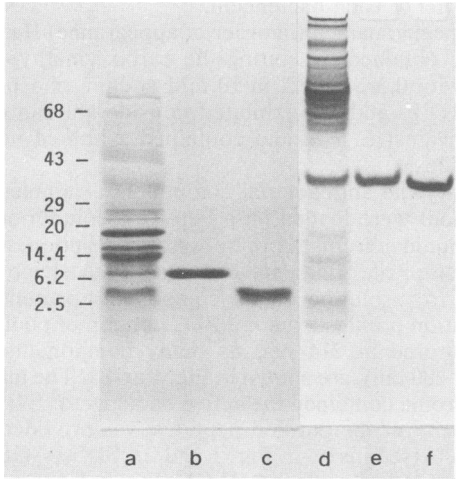

FIG. 3. PAGE of high-pressure liquid chromatography-purified bactenecins. Lanes a and d, Granule extracts (20 μg); lanes b and f, Bac-7 (6 μg); and lanes c and e, Bac-5 (6 μg). Samples were subjected to SDS-PAGE (left) and acid urea-PAGE (right) and stained with Coomassie brilliant blue. The numbers on the left margin are molecular weights (in thousands) of the proteins used for the SDS-PAGE calibration.

TABLE 1. Amino acid composition of Bac-5 and Bac-7[a]

Amino acid	mol/100 mol of:	
	Bac-5	Bac-7
Arg	20.1	28.1
Pro	46.8	47.6
Gly	2.9	5.3
Ile	11.4	6.8
Leu	2.8	6.9
Tyr	1.8	
Phe	14.2	5.3

[a] Samples of the two bactenecins were hydrolyzed at 110°C in 6 N HCl for 22, 48, and 72 h, and the amino acids in the hydrolysates were analyzed with an automatic amino acid analyzer. Data were obtained with 72-h hydrolysates.

gram-positive and gram-negative microorganisms. The two polypeptides exhibited considerable antibiotic activity in the microdilution susceptibility test against several gram-negative organisms, with MICs ranging from 6 to 200 μg/ml (Table 2). At the maximal concentration of Bac-5 and Bac-7 employed (200 μg/ml), *Staphylococcus aureus* and *Streptococcus agalactiae*, as well as *Proteus vulgaris*, were not susceptible to the two bactenecins. Finally, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were resistant to Bac-5 but susceptible to Bac-7.

The susceptibilities of the gram-negative bacteria tested appear to be dependent on the type of growth medium used. In fact, all the organisms are more vulnerable when grown in Mueller-Hinton broth than in Iso-Sensitest broth (Table 2), with MICs in the former medium being two- to fourfold lower than in the latter. The different susceptibilities, which have also been observed with other antibiotics, may depend on the effects of the medium composition on the length of the carbohydrate chain of the lipopolysaccharide (14).

To gain further insight into the antibacterial activities of Bac-5 and Bac-7, bactericidal assays were carried out with *E. coli*, *Salmonella typhimurium* ATCC 14028, and *K. pneumoniae* as test strains. The bactericidal activities of Bac-5 and Bac-7, used at concentrations ranging from 5 to 50 μg/ml, were both time and concentration dependent (Fig. 4

TABLE 2. Bacteriostatic activity of Bac-5 and Bac-7[a]

Organism and strain	MIC (μg/ml) in broth			
	Mueller-Hinton		Iso-Sensitest	
	Bac-5	Bac-7	Bac-5	Bac-7
Escherichia coli ATCC 25922	12	12	25	50
Salmonella typhimurium LT2	12	12	100	200
Salmonella typhimurium ATCC 14028	25	25	100	100
Klebsiella pneumoniae ATCC 13883	25	12	100	200
Enterobacter cloacae ATCC 13047	25	25	100	200
Proteus vulgaris ATCC 13315	>200	>200	>200	>200
Pseudomonas aeruginosa ATCC 7700	>200	50	>200	>200
Staphylococcus epidermidis ATCC 12228	>200	200	>200	200
Staphylococcus aureus ATCC 25923	>200	>200	>200	>200
Streptococcus agalactiae ATCC 13813	>200	>200	>200	>200

[a] Serial dilutions of the bactenecins in 50 μl of broth were pipetted into the wells of microdilution plates. Bacteria (1×10^4 to 2×10^4 CFU) in 50 μl of broth were then added. The final concentration of the peptide in the first well was 200 μg/ml. The plates were incubated at 37°C for 16 to 18 h. The assays were repeated at least three times.

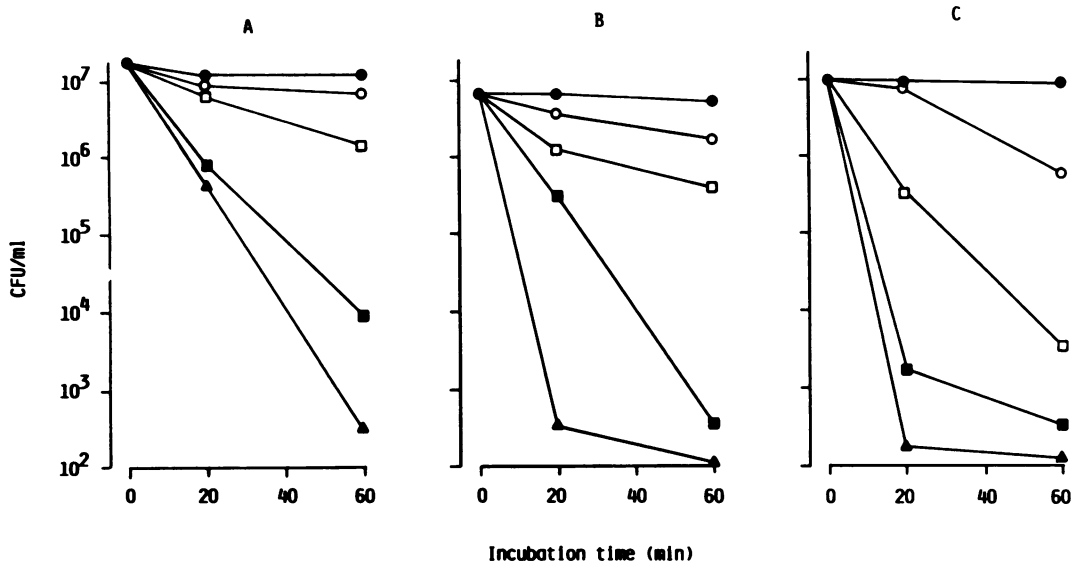

FIG. 4. Kinetics and dose-response effects of the bactericidal activity of Bac-5 on mid-log-phase cultures of *E. coli* ATCC 25922 (A), *Salmonella typhimurium* ATCC 14028 (B), and *K. pneumoniae* ATCC 13883 (C). Assays contained 1×10^6 to 2×10^6 CFU/0.2 ml of 100 mM NaCl in 10 mM sodium phosphate, pH 7.4; controls (●) contained TFA at a maximal final concentration of 0.005%. Bac-5 concentrations: 5 (○), 10 (□), 20 (■), and 50 (▲) μg/ml.

and 5). After 20 min of incubation, Bac-5 was more active on all three organisms tested than Bac-7, while at 60 min they were equally effective. With stationary-phase *E. coli*, the susceptibility to both Bac-5 and Bac-7 was about 10-fold lower, although the bacterial density in the bactericidal assays was greatly decreased (0.7×10^6 to 1.5×10^6 CFU/ml versus 5×10^6 to 10×10^6 CFU/ml) (data not shown). Finally, no bactericidal activity against *Staphylococcus aureus* could be detected even at Bac-5 or Bac-7 concentrations of 100 μg/ml (data not shown).

DISCUSSION

By employing only two chromatographic steps, we have resolved two new antibacterial cationic polypeptides from an extract of bovine neutrophil granules. The purified bactenecins arrested the growth of several gram-negative bacteria, as determined in microdilution susceptibility tests. Furthermore, when they were tested in bactericidal assays, the bactenecins efficiently killed *E. coli*, *K. pneumoniae*, and *Salmonella typhimurium*. Their activity thus resembles that of other cationic polypeptides, isolated from neutrophils of humans, rabbits, and guinea pigs (3, 4, 11–13). At variance with rabbit and human defensins, which are very sensitive to the ionic strength of the medium, bactenecins also maintain a considerable activity in vitro in media containing 100 mM NaCl.

The unique features of the bactenecins described here reside in their high content of proline in addition to arginine,

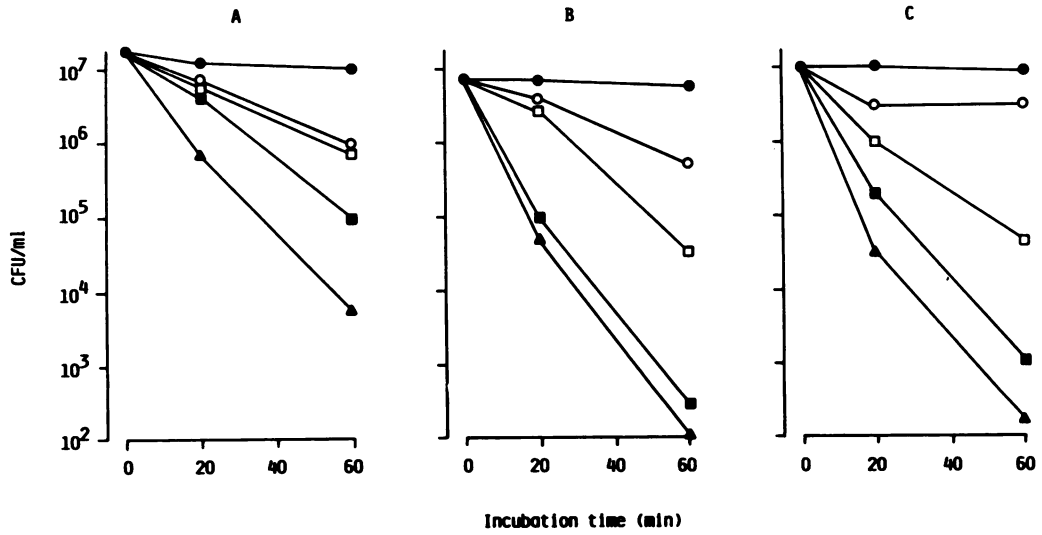

FIG. 5. Kinetics and dose-response effects of the bactericidal activity of Bac-7 on mid-log-phase cultures of *E. coli* ATCC 25922 (A), *Salmonella typhimurium* ATCC 14028 (B), and *K. pneumoniae* ATCC 13883 (C). Experimental conditions and symbols are as described in the legend to Fig. 4.

which confers the cationic nature on the polypeptides. In spite of their similarity in amino acid composition, the two bactenecins are different molecular entities. In fact, they lack immunological cross-reactivity, as tested with rabbit antiserum against either Bac-5 or Bac-7, and are processed from two different precursors, as revealed by Western blot (immunoblot) analyses (M. Zanetti et al., manuscript in preparation). Further, the two bactenecins have different N-terminal amino acid sequences, which are Arg-Phe-Arg-Pro-Pro-Ile-Arg-Arg-Pro-Pro and Arg-Arg-Ile-Arg-Pro-Arg-Pro-Pro-Arg-Leu for Bac-5 and Bac-7, respectively (unpublished data).

Bac-7 is also capable of neutralizing human herpes simplex virus type 1 (data not shown) and very likely corresponds to the bovine neutrophil antiviral peptide $IIIa_2\beta$ that we have previously purified by a more complex procedure from granule extracts (15). In fact, the two peptides have an identical molecular weight, a marked similarity in amino acid composition, and an identical N-terminal amino acid sequence, and they cross-react with the monoclonal antibody BP97 (7). By analogy with defensins (2), some bactenecins may thus exhibit both antibacterial and antiviral properties.

From the yield of the purification process of Bac-5 and Bac-7 from the granule extracts, we can estimate that 10^6 bovine neutrophils (0.35 μl of cell volume) should contain over 125 ng of each peptide. If only partial discharge (e.g., 10%) of the large-granule content should occur during phagocytosis, the intraphagolysosomal concentrations of Bac-5 and Bac-7 would largely exceed the efficient concentrations of the antibacterial peptides in vitro. In addition, the large granules may discharge the dodecapeptide bactenecin (8), whose intraphagolysosomal concentration has also been estimated to exceed the efficient concentration in in vitro bactericidal assays carried out with either *Staphylococcus aureus* or *E. coli*. These high potential intraphagolysosomal concentrations of bactenecins may provide a margin of safety for the antibacterial activity against possible antagonists such as proteases, low pH, or negatively charged macromolecules.

In conclusion, although the antibacterial (and antiviral) potential of the bactenecins has not yet been fully explored, on the basis of the data thus far collected on the potency and spectrum of their activity (5, 8, 15), it is very likely that they play a major role in host defense mechanisms in cattle.

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