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Antimicrobial Activity and Bacterial-Membrane Interaction of Ovine-Derived Cathelicidins

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Three ovine-derived cathelicidins, SMAP29, OaBac5mini, and OaBac7.5mini, were compared with respect to their antibacterial activities and interactions with membranes. SMAP29 was confirmed to be α-helical, broad spectrum, and able to disrupt both the outer and the cytoplasmic membranes at relatively low concentrations. In contrast, the two proline- and arginine-rich OaBac peptides had more-modest antibacterial activities, reduced levels of lipopolysaccharide binding, and a lesser ability to depolarize the cytoplasmic membrane, consistent with a cytoplasmic target.

In New Zealand alone, approximately 30 million lambs and 10 million sheep are processed annually, resulting in the availability of 40 million liters of ovine blood. Currently, this blood is used for low-value products, such as fertilizer, or is discarded as effluent. It would be more beneficial if the components of ovine blood (serum albumins, fibronectin, transferrin, antibodies, trypsin, etc.) were separated and sold as high-value products. The antimicrobial peptides present in ovine blood also have the potential to be used as commercial products, for example, as biopreservatives for chilled lamb products or in a topical cream for cuts and grazes (1). Before product development can occur, the properties of these peptides need to be better understood.

Virtually all living organisms produce cationic antimicrobial peptides for protection against invading microorganisms, and it is becoming clear that these peptides are an important component of the innate defenses of all species of life (8). Sheep have been relatively poorly characterized with respect to their antimicrobial peptides. In addition to two known defensins, they have eight known cathelicidins, including cyclic dodecapeptide, SMAP29, and Bac7.5 (10). For this work, three cathelicidin peptides were used. The first peptide, SMAP29, is an α-helical peptide (17) that was identified from ovine myeloid cDNAs based on its conserved cathelin region (2, 12). The corresponding mature SMAP29 peptide (RGLRRLGRKI AHGVKKYGPTVLRIIRIA-NH_2) has been synthesized and shown to have potent broad-spectrum activity (4, 18), although it has not been purified from ovine cells to date. The second peptide, OaBac5mini (RFRPPIRRPPIRPPFRPPFRPPVR-NH_2), was based on OaBac5, which is a 51-residue proline- and arginine-rich peptide that was first inferred based on a cDNA sequence (10). Since then, OaBac5, as well as three variants of OaBac5, has been isolated from sheep neutrophils (1a, 16). OaBac5 is made up of a 6-residue N terminus followed by two copies of a 16-residue repeat and a 5-residue C terminus. OaBac5mini, a truncated version of OaBac5, is made up of the 6 N-terminal residues, one copy of the 16-residue repeat, and the first 2 residues of the second repeat. This truncation was chosen because the full-length molecule could not be synthesized, and it has been shown that the bovine peptide Bac7 retained its activity when it was similarly truncated (15). Like OaBac5, OaBac7.5 is rich in Pro and Arg and was predicted from ovine DNA (10). However, OaBac7.5 (predicted to be 60 residues long) has been isolated from sheep neutrophils only as a truncated, 29-amino-acid C-terminal peptide, OaBac7.5mini (RRIPRPILLPWRPPRPIPRPQPQPIPRWL-NH_2 [1a]). The three ovine-derived peptides were synthesized by *N*-(9-fluorenyl)methoxy carbonyl solid-phase peptide synthesis with a model 432A synthesizer (Applied Biosystems Inc., Foster City, Calif.) at the University of British Columbia Nucleic Acid/Protein Service facility. The purities of the peptides were confirmed to be at least 99% by high-performance liquid chromatography and mass spectrometry analysis. SMAP29 was constructed as a 28-amino-acid amidated peptide because this is thought to be its natural form.

The MICs of the peptides for a variety of organisms were determined by a modified broth dilution method (20) and were the modes of five assessments done on separate days (Table 1). SMAP29 was confirmed to have potent activity against all organisms tested (with MICs of 0.125 to 4 μg/ml). OaBac5mini had potent activity against gram-negative bacteria (MICs, 0.125 to 8 μg/ml) but weak activity against gram-positive bacteria and the yeast *Candida albicans* (MICs, 16 to 64 μg/ml). OaBac7.5mini had relatively weak activity against all of the test organisms except the outer-membrane-weakened, antibiotic-supersusceptible strains *Escherichia coli* DC2 and *Salmonella enterica* serovar Typhimurium MS4252S (a *phoPQ* mutant) and the rough-lipopolysaccharide (LPS) strain *E. coli* UB1005. Measurements of bacterial growth and survival in the presence of twofold the MIC of each peptide demonstrated that SMAP29 (at 4 μg/ml) was rapidly bactericidal, reducing cell numbers by 6 log orders in the first 5 min. In contrast, OaBac5mini (at 4 μg/ml) was bacteriostatic, whereas OaBac7.5mini (at 32 μg/ml) slowed growth only over the first 2 h.

Circular dichroism (CD) spectroscopy was carried out for

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TABLE 1. MICs of ovine antimicrobial peptides for various microorganisms

Organism	MIC (μg/ml)		
	SMAP29	OaBac5mini	OaBac7.5mini
Gram-negative bacteria			
E. coli 0111	2	2	16
E. coli UB1005; rough K-12 strain	0.125	0.125	8
E. coli DC2; antibiotic-supersusceptible mutant	0.125	0.125	2
E. coli O157:H7	2	8	32
S. enterica serovar Typhimurium 14028s	0.25	0.5	32
S. enterica serovar Typhimurium MS4252S (*phoPQ* mutant; defensin supersusceptible)	0.125	0.125	2
Pseudomonas aeruginosa PAO1	4	4	16
P. aeruginosa Z61; antibiotic-supersusceptible mutant	1	8	32
Gram-positive bacteria			
Staphylococcus aureus NCTC 4163	1	32	64
S. aureus MRSA R147	0.5	64	32
S. aureus 1056 MRSA	4	16	>64
Staphylococcus epidermidis clinical isolate	0.25	16	32
Enterococcus faecalis ATCC 29212	2	32	64
Yeast			
C. albicans 105	2	32	64
C. albicans 3153A	4	16	>64

each peptide in three different solutions (Fig. 1). The CD spectra of SMAP29 in aqueous buffer displayed a negative band at around 200 nm, indicating that the structure is random. However, in 50% trifluoroethanol, hydrophobic conditions, and in 10 mM lyso-PC/lyso-PG (1:1) conditions that mimicked those associated with bacterial anionic membranes, SMAP29 had clear minima at around 206 and 230 nm, confirming that the peptide adopts an α-helical structure in these environments. The structures were more difficult to assign based on the CD spectra of OaBac5mini and OaBac7.5mini. Both appeared to be typical of a polyproline type II helix under membrane-mimicking conditions, with a minimum at 202 nm (5). OaBac5mini had a more pronounced spectrum than OaBac7.5mini, possibly because the latter contained additional contributions from other turn structures, which would tend to broaden the minimum that defines the polyproline type II helix. The wavelength shift of the minimum was probably related to the change in solvent polarity (water → trifluoroethanol → lipid) among the different suspensions.

Cationic peptides with activities against gram-negative bacteria interact with LPS on the surface of the outer membrane as a precursor to self-promoted uptake (8). To determine whether the test peptides bound to *E. coli* UB1005 LPS, the ability of the peptides to displace dansyl polymyxin B (DPX) from LPS was examined (13) (Table 2). DPX fluoresces strongly only when it is bound to LPS, permitting its displacement to be assessed by the decrease in fluorescence. Of the three peptides, SMAP29 displaced the most DPX and was able to cause 50% maximal displacement at the lowest concentration, indicating that it had the highest affinity for LPS. OaBac7.5mini was able to displace more DPX than OaBac5mini; however, it appeared to have a lower apparent affinity for LPS.

The ability of the test peptides to permeabilize the outer membrane of *E. coli* UB1005 was assessed by measuring their ability to promote the uptake of 1-*N*-phenylnapthylamine (NPN) into intact cells (11). All of the peptides were able to facilitate the uptake of NPN to similar degrees (data not

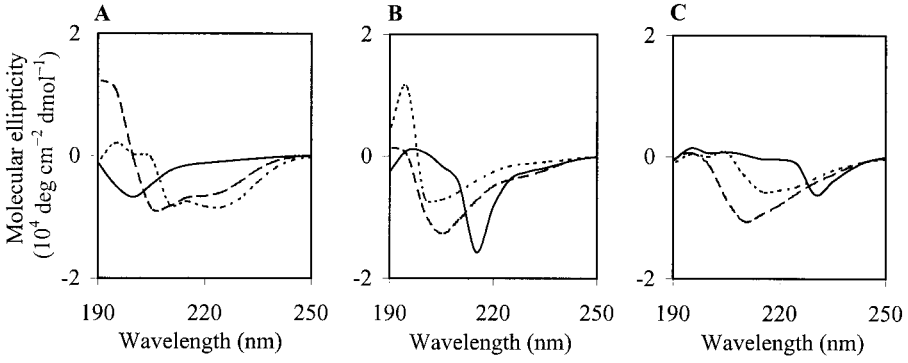

FIG. 1. CD spectra of 25 mM SMAP29 (A), 25 mM OaBac5mini (B), and 25 mM OaBac7.5mini (C) in 25 mM phosphate buffer (solid line), 50% 2,2,2-trifluoroethanol (dashed line), and 10 mM lyso-PC–lyso-PG (dotted line). deg, degrees.

TABLE 2. Abilities of ovine peptides to bind to *E. coli* LPS as determined by the DPX displacement assay[a]

Peptide	I_{max} (%)	I_{50} (μg/ml)
SMAP29	90	0.78
OaBac5mini	58	2.32
OaBac7.5mini	72	5.00

[a] I_{max}, percentage of DPX displaced relative to the total amount of DPX bound; I_{50}, concentration of peptide required to give half of the maximum DPX displacement.

shown). The peptide concentration causing half the maximal uptake of NPN was 0.4 to 0.5 μg/ml for each of the three peptides.

After passing through the outer membranes of gram-negative bacteria, antimicrobial peptides are able to interact with bacterial cytoplasmic membranes and permeabilize and/or traverse these membranes (7). This process was assessed by measuring the ability of peptides to depolarize the cytoplasmic membrane of the *E. coli* mutant DC2, whose outer membrane is permeable (20), by using the membrane potential-sensitive fluorescent dye $DiSC_35$ (3,3′-dipropylthiadicarbocyanine iodide) (Fig. 2). $DiSC_35$ inserts into the cytoplasmic membrane under the influence of the membrane potential gradient and quenches its own fluorescence. After the addition of a permeabilizing peptide that disrupts membrane potential, the dye is released, leading to an increase in fluorescence. Of the three test peptides, only SMAP29 caused substantial depolarization of the cytoplasmic membrane at concentrations similar to the MIC for *E. coli* DC2. In contrast, the other peptides were relatively ineffective even above the MIC.

The potent broad-spectrum activity of SMAP29 is already well documented (4, 18), but the activities of OaBac5mini and OaBac7.5mini had not been investigated previously. OaBac5mini was almost as active as SMAP29 against gram-negative bacteria, but otherwise these peptides were considerably less active than SMAP29. As predicted, the truncated OaBac5mini had antimicrobial activity similar to that previously determined for the full peptide, indicating that the full molecule was not required for the retention of activity (16). Conversely, compared to the bovine proline- and arginine-rich analogue, Bac5 (6), OaBac5mini was more active against both gram-negative and gram-positive bacteria. OaBac5 and Bac5 differ in only 5 of their 43 residues; however, 2 of these differing amino acids are Arg residues in the OaBac5 sequence whereas they are Tyr and Phe residues in the Bac5 sequence. These differences make Bac5 more hydrophobic and less cationic than OaBac5, possibly accounting for the differences in activity. The activity of OaBac7.5 has not been investigated; however, the activity of its bovine analogue (64% homologous), Bac7, against gram-negative bacteria is similar to that of OaBac7.5mini (6), but Bac7 is not as active as OaBac7.5mini is against gram-positive bacteria. Like the bovine peptides (14), these Pro- and Arg-rich ovine peptides appear to adopt a polyproline type II extended helix structure.

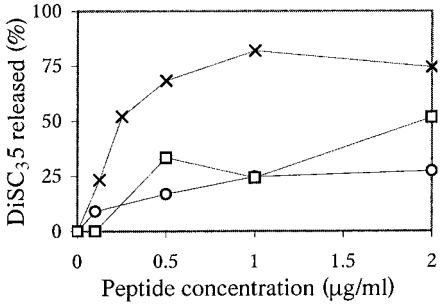

FIG. 2. Release of $DiSC_35$ dye from the cytoplasmic membranes of *E. coli* DC2 cells caused by SMAP29 (×), OaBac5mini (○), and OaBac7.5mini (□). The amount of $DiSC_35$ released is given as a percentage of the maximum release of $DiSC_35$ caused by gramicidin.

The first step in the interaction between antimicrobial peptides and bacterial membranes is thought to be the binding of the positively charged peptides to the negatively charged LPS on the outer bacterial surface. The results of the present study show that the test peptides all bound to LPS, with SMAP29, the most potent peptide, having the highest affinity. However, it should be noted that this enhanced LPS binding did not correlate with enhanced activity against gram-negative bacteria, as OaBac5mini had activity similar to that of SMAP29 against gram-negative bacteria, which contain LPS, and had inferior activity only against gram-positive bacteria, which lack LPS. Our results for SMAP29 were, however, consistent with those of a previous study that indicated that SMAP29 has two LPS binding sites, one at each end of the molecule, and that these sites bind cooperatively (19). All three peptides caused similar enhancements of the uptake of NPN into *E. coli* cells despite their different antimicrobial activities, showing that they made the outer membrane more permeable. The simplest explanation for this finding is that passage across the outer membrane does not limit antimicrobial activity for these peptides.

Once the peptides have passed through the outer membranes of bacterial cells, they are able to interact with the cytoplasmic membranes. All three peptides caused some depolarization of the cytoplasmic membrane, confirming that they traversed the outer membrane. However, of the test peptides, only SMAP29 at its MIC (0.125 μg/ml) caused substantial depolarization of the cytoplasmic membrane, indicating that cytoplasmic membrane disruption might be involved in its mode of action. In contrast, OaBac5mini and OaBac7.5mini caused relatively low cytoplasmic membrane depolarization at their MICs (0.125 and 2 μg/ml, respectively).

The differences between the behavior of the proline- and arginine-rich peptides and that of the α-helical SMAP29 were also illustrated by the kill curve experiments. SMAP29 reduced the number of viable cells dramatically in a very short time period and caused a decrease in the optical density of the solution, which indicates that cell lysis occurred. This finding is consistent with the theory that the higher level of membrane depolarization caused by SMAP29 leads to the loss of the proton gradient, resulting in the leakage of essential molecules and cell death. A previous study using different techniques also showed that SMAP29 caused outer and inner membrane permeabilization (18).

Unlike SMAP29, OaBac5mini and OaBac7.5mini did not cause a decrease in viable cell numbers during 2 h of incubation. Instead, compared with what occurred with the untreated control, these peptides limited the increase in viable-cell numbers and the optical density of the solution, which indicates

that they inhibited the division of the cells. For these peptides, the minimal inner membrane depolarization was not enough to cause cell death; instead, it appeared to be an intermediate step in the process. The peptides probably passed through the cytoplasmic membrane and interacted with cellular contents, like PR-39, a porcine proline- and arginine-rich peptide that kills bacteria by stopping protein and DNA synthesis (3).

In addition to the cathelicidin peptides studied here, one other α-helical peptide (SMAP34), two other proline- and arginine-rich peptides (OaBac6 and OaBac11), and an amphipathic loop peptide held in place by a disulfide bond (OaDode) have been predicted from ovine cDNA (10). Two β-defensins have also been identified in sheep, but unlike bovine β-defensins, these β-defensins are not present in neutrophils (9). This finding indicates that there are a variety of peptides that are able to fight infections in the animal and that these peptides use at least two different mechanisms of action (inner membrane depolarization and interaction with cytoplasmic contents). The use of peptides with different mechanisms may be advantageous in decreasing the likelihood of resistance and promoting synergistic interactions. This finding also indicates that if a mixture of antimicrobial peptides is isolated from ovine blood and used in a commercial product (chilled-meat biopreservative or topical antiseptic cream), peptides utilizing different mechanisms should be present to provide "insurance" of the product's effectiveness against a wide range of pathogens.

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