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ORIGINAL RESEARCH PAPER

Antimicrobial fragments of the pro-region of cathelicidins and other immune peptides

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Abstract In addition to the numerous cathelicidin peptides that are associated with the antimicrobial activity exhibited by a crude extract from ovine blood, a further three peptides with antimicrobial activity have been identified. These were part of the precursor cathelin domain of cathelicidins, a large fragment of platelet factor 4 and a small peptide similar to signal peptide of the T-cell glycoprotein CD4 precursor. Fragments of proteins that are involved in protecting the host from infection may have a secondary purpose as antimicrobial agents once they have carried out their primary purpose and are cleaved the main protein.

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Introduction

The aim of this research was to investigate further the components responsible for the antimicrobial activity displayed by a crude extract from ovine blood. This extract is of interest because it has the potential to be a high-value product produced from the large volumes of ovine blood that are currently a waste product from the lamb slaughtering industry. This extract could be used as a biopreservative for chilled lamb products or in a topical cream for cuts and grazes (Anderson and Yu 2004).

Our previous work showed that the majority of the antimicrobial activity of the ovine crude extract was due to a group of proline/arginine-rich cathelicidin peptides (Anderson and Yu 2003). Cathelicidins are synthesised in maturing myeloid cells in the bone marrow as pre-pro-peptides. The N-terminal pre-pro-reigion is highly conserved between species (Storici et al. 1992). This pro-piece is very similar to a porcine cysteine-protease inhibitor, cathelin, hence the name cathelicidins (Zanetti et al. 1995). The pre-region is removed by signal peptidase and then the pro-peptides are stored in the neutrophil granules. In contrast to the pre-pro-regions, the C-terminal regions of cathelicidins are highly

variable. The molecules are not active until the pro-piece is proteolytically removed. This cleavage is carried out in the neutrophils, by neutrophil elastase (Panyutich et al. 1997; Zanetti et al. 1993) or proteinase 3 (Sorensen et al. 2001), before the active peptides are released into the extracellular fluid.

Eight ovine genes encoding for seven different cathelicidins have been identified: two a-helical peptides, SMAP29 and SMAP34 (also called OaMAP28 and OaMAP34); four arginine/proline-rich extended structures, OaBac5, OaBac6, OaBac7.5, and OaBac11; and two copies of the dodecapeptide OaDode (Huttner et al. 1998). Prior to our work, only two variants of the predicted peptide OaBac5 had been isolated from ovine neutrophils (Shamova et al. 1999). In addition to these, we isolated the originally predicted OaBac5 and another OaBac5 variant, the C-terminus of the predicted OaBac7.5, and various truncated forms of OaBac11. Since our work, it has been shown that the human cathelicidin LL-37 is also processed into various smaller peptides with antimicrobial activity (Murakami et al. 2004).

The hypothesis of this work was that the ovine crude extract contained other components, not only the proline/arginine-rich cathelicidins we had already isolated, which were responsible for part of the antimicrobial activity. Here we report the purification of three protein fragments that had antimicrobial activity, including a fragment of the cathelicidin precursor.

Materials and methods

Crude extraction

A crude antimicrobial extract from the neutrophil granules of ovine blood was produced as previously described (Anderson and Yu 2003; Yu et al. 2001). Briefly, the blood was mixed with 10% (w/v) sodium citrate to stop coagulation and was filtered to remove large solids, such as wool. The red blood cells were lysed by the addition of 0.83% (w/v) ammonium chloride solution to the blood at a ratio of 3:1. The white blood cells were collected by centrifugation (700 *g*, 15 min, 4°C), resuspended in PBXS buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), and sonicated to release the neutrophil granules. These granules were collected (27,000 *g*, 40 min, 4°C), suspended in 10% (v/v) acetic acid, and mixed overnight at 4°C to extract the antimicrobial peptides. The solution containing the peptides was separated from the granules (27,000 *g*, 20 min, 4°C), freeze-dried, and suspended in 0.01% (v/v) acetic acid.

Cationic-exchange chromatography was used to separate the cationic molecules from the non-cationic molecules in the crude extract. Fractions (5 ml) corresponding to the peaks were collected, freeze-dried and suspended in 0.01% (v/v) acetic acid. RP-HPLC was used to separate the components in the active fraction cationic-exchange fractions. The fractions were collected manually, freeze-dried and suspended in 0.01% acetic acid.

Peptide activity

The cationic-exchange fractions and HPLC fractions were tested for activity using the radial diffusion plate assay method (Steinberg and Lehrer 1997). The test cultures used were *Escherichia coli* O111, *Staphylococcus aureus* NCTC 4163 and *Candida albicans* 3153A. These cultures were chosen to represent Gram-negative bacteria, Gram-positive bacteria and yeast, respectively. For each plate assay a positive control and negative control was included. The positive control was a common antibiotic; 1 mg polymyxin B/ml for *E. coli* O111, 1 mg nisin/ml for *S. aureus* NCTC 4163 and 10 mg nystatin/ml for *C. albicans* 3153A. The negative control was 0.01% acetic acid because this was what was used to suspend the samples. The protein concentrations and minimal inhibitory concentrations could not be determined due to the limited amount of each peptide purified.

Peptide characterisation

The mass spectroscopy and N-terminal sequencing of the peptides in RP-HPLC Chromatograph A (See Fig. 2 below) were performed by Protein Microchemistry Facility, Otago University, Dunedin, New Zealand. Samples for MALDI-TOF-MS were prepared by mixing the peptide samples (0.5 µl, from 50 µl total volume HPLC fractions) and the matrix [0.5 µl of 10 mg α-cyano-4-hydroxycinnamic acid/ml

in 0.1% TFA, 60% (v/v) acetonitrile] directly on a stainless steel slide, and leaving them to dry at room temperature. Mass data were collected at near threshold laser fluorescence in the positive ion mode, with a linear instrument (Finnigan Lasermat 2000, from Thermo Bioanalysis). Where internal calibration was used, this was added to the sample/matrix mixture as indicated. The sample [diluted 1:1 from previous dilution in 50% (v/v) acetonitrile] was directly infused at 3 µl/min into the Finnigan LCQ Deca Mass Spectrometer, using the Electrospray Ionisation Probe. Spray voltage was set to 5 kV, sheath gas (N_2) was set to approximately 33 units and the capillary temperature was 220°C. Data was collected over a series of scans and these averaged to produce the spectrum observed. For the N-terminal sequencing, Automated Edman peptide sequencing was carried out on a glass fibre disk using an Applied Biosystems Procise 492 Protein Sequencer with pulsed liquid trifluoroacetic acid delivery.

The mass spectroscopy and N-terminal sequencing of the peptides in RP-HPLC Chromatograph B (See Fig. 3) were performed by School of Biological Sciences, University of Auckland, New Zealand. The mass spectrometry was carried out using an Applied Biosystems Voyager DE PRO MALDI-TOF-MS. The ion-acceleration potential used was 20 kV and for each sample the "100 shots" mode was used to acquire data within the 500–8,000 Da range of positive polarity. For the N-terminal sequencing, automated Edman degradation using an Applied Biosystems 470 Procise Protein Sequencing System was used.

Results

The ovine neutrophil crude extract was passed through a weak cationic exchange column to separate the cationic molecules from the non-cationic molecules (Fig. 1). The fractions were tested for antimicrobial activity using the radial diffusion plate assay method (Steinberg and Lehrer 1997). The non-cationic fractions (F1 and F2) did not display antimicrobial activity; whereas, the cationic fractions (F3 and F4) were active against the three test organisms, *E. coli* 0111, *S. aureus* NCTC 4163 and *C. albicans* 3153A.

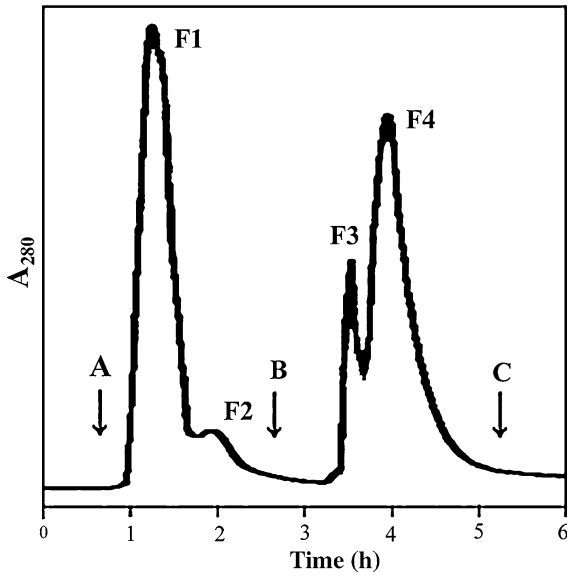

Fig. 1 Ion-exchange chromatograph for the addition of the ovine neutrophil crude extract to a weak cationic exchange column (Macro-Prep CM resin, BioRad Laboratories, California). At time 'A' the sample was added. The cationic molecules bound to the anionic resin and the non-cationic molecules were washed through with 25 mM ammonium acetate. At time 'B' the running buffer was changed to 10% (v/v) acetic acid to elute the cationic molecules. At time 'C' the running buffer was changed to 20% (v/v) ethanol to wash the column

The cationic fractions (F3 and F4) were pooled due to their inadequate separation and further purified using RP-HPLC. Initially, an acetonitrile gradient of 20–40% (v/v) over 30 min was used (Fig. 2). This profile was similar to that we have already described for the separation of various ovine proline/arginine-rich cathelicidins (Anderson and Yu 2003). Of the 12 peaks collected (A2–A13), nine displayed antimicrobial activity. The molecular weights of the peaks were determined, and A5 (mass 8,300 Da) was chosen for N-terminal sequencing because its mass did not match those expected for proline/arginine-rich cathelicidins. However, the N-terminal sequence of A5 matched part of ovine platelet factor 4 (Table 1).

Plate assays showed that the fraction collected at the void volume of the initial RP-HPLC fractionation also displayed antimicrobial activity. Therefore, a less hydrophobic acetonitrile gradient of 0–20% over 30 min was used to separate these void volume components (Fig. 3). Of the 36 peaks collected (B1–B36), 13 displayed antimicrobial activity.

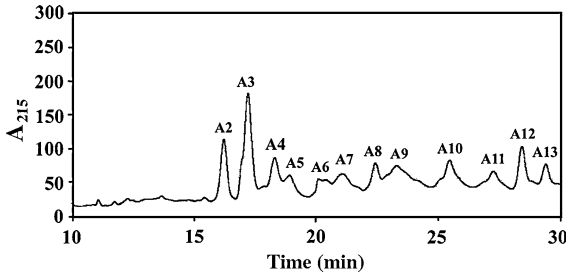

Fig. 2 RP-HLPC Chromatograph A of the cationic fraction of the ovine crude extract using a Dionex HPLC system with a Phenomenex Jupiter Proteo column. Buffer A contained 5% (v/v) acetonitrile and 0.1% trifluoroacetic acid (TFA). Buffer B contained 95% (v/v) acetonitrile and 0.1% TFA. A gradient from 20% to 40% of Buffer B over 30 min was used

The molecular weights of the six most active and well separated peaks were determined, and B18 and B24 were chosen to be further characterised. B18 (3,185 Da) was selected because it was separated well from the other peaks and because it was the only peak of those investigated that had a mass in the range expected for antimicrobial peptides (3–5 kDa). The N-terminal sequence of B18 was similar to part of the cathelin-like domain of cathelicidins (Table 1). B24 (mass 1,126 Da) was chosen because it had the highest antimicrobial activity even though it was a small peak and therefore had a relatively low concentration. The N-terminal sequence of B24 matched part of the signal peptide of the T-cell surface glycoprotein CD4 precursor (Table 1).

Discussion

One of the antimicrobial peptides activity was identified as a fragment of ovine platelet factor 4 (PF4). This isolated peptide did not contain the first nine N-terminal residues of the full sheep PF4 sequence (Shigeta et al. 1991); however, the molecular weight of the isolated peptide (8,300 Da) matched that predicted for the remaining 76 residues. PF4 is also known as CXCL4 (chemokine [CXC motif]

Table 1 Comparison of the N-terminal sequences of ovine crude extract peptides to related proteins

Peptide	Sequence
A5 N-terminus	SLPADSEGGE……
	: : : : : : : : : :
Ovine platelet factor 4	XSSLPAASVSLPADSEGGEEEDLQCVCLKTSGIHPRHISSLEVIGAGL
	HCPSPQLIATLKTGRKICLDQQNPLYKKIIKRLLKN
B18 N-terminus	LSLY-EAVLYAVDT……
	: : : : : : : : : : : : : :
SMAP29 prepro-peptide	METQRASLSLGRRSLWLLLLGLVLASARAQALS-YREAVLRAVDQLNE
	KSSEANLYRLLELDPPPKQDDENSNIPKPVSFRVKETVCPRTSQQPAE
	QCDFKENGLLKECVGTVTLDQVGNNFDITCAEPQSVRGL**RRLGRKIAH**
	GVKKYGPTVLRIIRIAG
B24 N-terminus	VLQLAL……
	: : : : : : :
Human T-cell surface glycoprotein CD4 precursor	MNRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKS
	IQFHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNFPLII
	KNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLE
	SPPGSSPSVQCRSPRGKNIQGGKTLSTWTCTVLQNQKKVEFKIDIVVL
	AFQKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKS
	WITFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSGNLT
	LAEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKREK
	AVWVLNPEAGMWQCLLSDSGQVLLESNIKVLPTWSTPVQPMALIVLGG
	VAGLLLFIGLGIFFCVRCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQ
	KTCSPI

The signal peptides are underlined and the mature cathelicidin is given in bold text

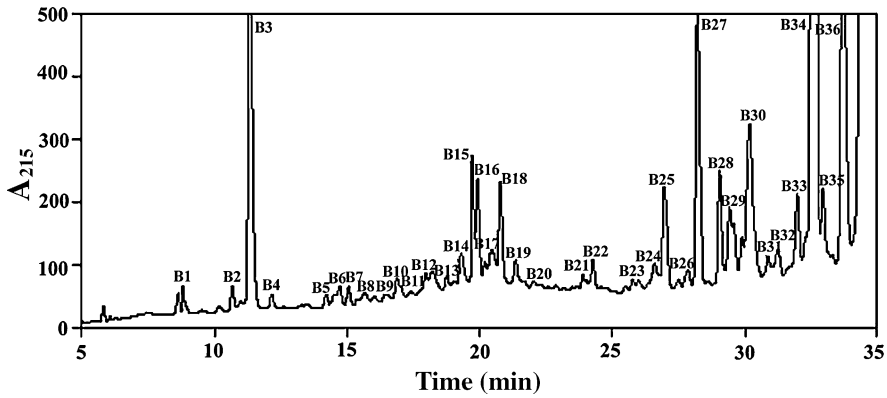

Fig. 3 RP-HLPC Chromatograph B of the cationic fraction of the ovine crude extract using a Dionex HPLC system with a Phenomenex Jupiter Proteo column. Buffer A contained 5% (v/v) acetonitrile and 0.1% trifluoroacetic acid (TFA). Buffer B contained 95% (v/v) acetonitrile and 0.1% TFA. A gradient from 0% to 20% from Buffer B over 30 min was used

ligand 4) and it accounts for approximately 25% of the protein in the platelet α-granule (Slungaard 2005). Our result showing the antimicrobial activity of ovine PF4 against *E. coli* and *S. aureus* agrees with the results of Tang et al. (2002) who demonstrated antimicrobial activity of human PF4 against *E. coli* and *S. aureus*, and also against *Cryptococcus neoformans* and *Candida albicans*. Other CXC chemokines have been shown to display antimicrobial activity (Cole et al. 2001). Like the active CXC chemokines, PF4 has a cationic C-terminus, and is structurally similar to defensins (Durr and Peschel 2002); therefore it is likely to have a similar membrane destabilising mechanism as defensins.

The large size of the PF4 peak in the RP-HPLC chromatograph indicates a relatively high contamination of the neutrophil crude extract with platelets. The platelet purification process is similar to that of neutrophils (Yeaman et al. 1992) so it is likely that the neutrophils and platelets co-pelleted due to suboptimal centrifugation conditions. This could be rectified by optimising the centrifugation conditions and checking the purity of the neutrophils using staining and microscopy prior to the peptide extraction. However, due to the antimicrobial properties of the components extracted from the platelets it would be best to produce a combined neutrophil and platelet crude extract to maximise the antimicrobial activity.

Another of the peptides with antimicrobial activity isolated was a fragment of the cathelin-like precursor of cathelicidins. It was originally thought that this precursor only had the function of suppressing the antimicrobial activity of the cationic peptide until it was required, and that it may be involved in targeting and/or assisting the folding of the antimicrobial peptide (Zanetti et al. 1995). A study using a recombinant human cathelin protein showed that the cathelin domain was able to inhibit the activity of protease cathepsin L (Zaiou et al. 2003). This study also showed that when cleaved the cathelin domain displayed antimicrobial activity, whereas the full molecule did not. These findings suggest that after proteolytic cleavage the cathelin domain can contribute to host defence by inhibiting bacterial growth and limiting tissue damage mediated by cysteine proteinase. The peptide isolated in this work was only a small fragment of the cathelin domain (molecular weight 3,185 Da compared to that of full signal peptide15,000 Da) yet it still inhibited the growth of the test bacteria. This shows that the complete cathelin domain is not required for antimicrobial activity.

The third peptide characterised was a fragment of the signal peptide of the T-cell surface glycoprotein CD4 precursor (Maddon et al. 1986). CD4 is a type I integral glycoprotein of 55 kD. CD4 is translated on the membrane-bound polysomes and co-translationally inserted into the endoplasmic reticulum where the 25 amino acid signal sequence in cleaved (Bowers et al. 1997). CD4 plays a role in both thymocyte development and T-cell activation, and it may also act as an adhesion molecule. CD4 is the primary cell surface receptor for HIV and SIV. As a consequence the number of CD4+ T-cells in AIDS patients is greatly

reduced, which in turn reduces their ability to create an immune response to infection. Peak 24 may be a fragment of the signal peptide of the precursor of the CD4 protein. This indicates that the signal peptide itself may also play a role in fighting infections.

This research showed that along with the proline/arginine-rich cathelicidins already purified, there are a number of small peptides present in the ovine neutrophil crude extract that also have antimicrobial properties. One of these peptides was similar to the cathelicidin peptide precursor and another may have been part of the signal peptide of the T-cell surface glycoprotein CD4. This indicates that these signal peptides and precursors to protein and peptides that are involved in protecting the host from infection, may have a secondary purpose as antimicrobial agents once they have carried out their primary purpose and are cleaved from the main protein. Additionally a large fragment of PF4 that displayed antimicrobial activity was also isolated confirming its role as an antimicrobial agent. The presence of numerous peptides that may have diverse mechanisms of action to inhibit bacterial growth is advantageous because if a microorganism is resistant to one mechanism it may still be susceptible to another.

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