ANTIMICROBIAL PEPTIDES ISOLATED FROM OVINE BLOOD NEUTROPHILS

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Biotechnology

at Massey University, Palmerston North, New Zealand.

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2005

ABSTRACT

The aim of the research presented in this thesis was to investigate the properties of the antimicrobial peptides found in ovine blood, in order to assess their potential as a high-value product. Due to the large number of lambs and sheep that are slaughtered New Zealand (approximately 25 million lamb and 5 million sheep per year), there are considerable volumes of ovine blood available for processing (approximately 40 million litres per year). Currently this blood is dried and sold as a low value product. The first objective of this research was to purify and characterise the antimicrobial peptides isolated from ovine neutrophils. A number of proline/arginine-rich peptides, as well as two small fragments of larger proteins, that displayed antimicrobial activity were identified. The second objective of this research was to investigate the mechanism of action of ovine antimicrobial peptides. For this investigation, three ovine peptides, α-helical SMAP29 and proline/arginine-rich OaBac5mini and OaBac7.5mini, were synthesised. Of these, SMAP29 was the most potent. The three peptides all bound Gram-negative bacterial LPS and caused the outer membrane to be permeabilised. SMAP29 caused significant depolarisation of the cytoplasmic membrane that led to cell lysis. However, the other two peptides only caused slight depolarisation of the cytoplasmic membrane, which indicates that they probably passed through the membrane to interact with the inner cellular contents. The third objective of this research was to investigate the morphological changes to bacterial cells induced by the ovine antimicrobial peptides. Transmission electron microscopy and atomic force microscopy confirmed that SMAP29 caused significant damage to the membranes of bacterial cells and induced cell lysis; whereas, OaBac5mini caused minor alterations to the bacterial membranes but did not induce cell lysis. The fourth objective of this research was to determine the effect of the environmental conditions on the activity of the peptides. The peptides were very stable over a range of pH values and when heated to temperatures up to 80°C. The activity of the peptides decreased slightly in the presence of monovalent cations and was inhibited by the presence of divalent cations. The peptides were significantly more active in combination than individually, and they were strongly synergistic with polymyxin B, a peptide antibiotic. The final objective of this research was to develop a pilot-scale extraction process for the isolation of antimicrobial peptides from ovine blood. The laboratory-scale process was simplified and adapted to design a process that could be used industrially. The crude pilot-plant extract was active against a broad-range of food pathogens and disease causing organisms. The antimicrobial peptides found in ovine blood have the potential to be used as biopreservatives for chilled lamb products, or in a topical cream for cuts and grazes; therefore it is recommended that further research is carried out to investigate the above applications and, if successful, the feasibility of commercialising the technology.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisors. Dr Pak-Lam Yu, thank you for taking a keen interest in my project and for teaching me all I need to know to be a successful researcher. Dr Brian Wilkinson, thank you for being around when I needed that extra bit of help or advice. Professor Ian Maddox, thank you for joining the team to help me with the preparation of this manuscript.

I would also like to thank Professor Robert Hancock and his team for allowing me to visit their laboratory at the Department of Microbiology and Immunology, University of British Columbia, for three months, and for supervising and assisting with my bacterial membrane interaction experiments.

This work was made possible by the financial support I received from Meat and Wool New Zealand (formerly MeatNZ), in the form of both a doctoral scholarship and project funding. The project was also partially funded by the Massey University Research Fund (MURF), and my research trip to UBC was funded by the C. Alma Baker Trust.

This work was made easier by help I received from numerous people including the ITE technical staff, especially Anne-Marie Jackson and Mike Sahayam, and the staff of Feilding Lamb Packers, who collected the sheep blood for my experiments. I also received valuable help from the undergraduate and foreign-intern students that assisted on various parts of this project, including David Houlding (laboratory extraction process), Adi Sugiarto (RP-HPLC), Marie Bourin (crude extract MICs) and Andrew Lister (pilot-scale extractions). I received assistance from Aaron Hicks (Institute of Veterinary, Animal and Biomedical Sciences) to prepare the TEM samples, HortResearch to image the TEM samples, and Associate Professor Richard Haverkamp to image the AFM samples.

Finally, I would like to thank family and friends who helped keep me sane throughout this whole process. Other postgrads, especially Craig, Stephen, Roland and Anna, it always helped to know that there were others who shared the same, or worse, difficulties – Good luck to you all. Regan, thank you for caring enough to wade through this thesis to find the spelling and grammatical mistakes – a best friend who doubles as a proof-reader, what more could I ask for? Dad, I would never have made it this far without the support of you and "The Anderson Trust" – I think I was the best fed undergraduate student in town. And finally, Peter, there are not words to describe how much I appreciate you – I look forward to the future we will spend together.

I dedicate this thesis to my mother, who I know would have been proud. Her encouragement, support and love will be with me always.

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LIST OF ABBREVIATIONS

AFM	atomic force microscopy
Bac	bactenicin
BMAP	bovine myeloid antimicrobial peptide
BSA	bovine serum albumin
CD	circular dichroism
cDNA	complementary DNA
CFU	colony forming units
ChBac	*Capra hircus* bactenecin
$DiSC_35$	3,3-dipropylthiacarbocyanine
DNA	deoxyribonucleic acid
DPX	dansyl polymyxin B
EDTA	ethylenediaminetetraacetic acid
FIC	fractional inhibitory concentration
HLPC	high performance liquid chromatography
I_{50}	concentration of peptide required to displace half the of the maximum displacement amount of DPX from LPS
IFN-γ	interferon-γ
IL-12	interleukin-12
I_{max}	maximum percentage of DPX that could be displaced from LPS by the peptides
LPS	lipopolysaccharide
LTPs	lipid transfer proteins
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
MAP	myeloid antimicrobial peptides
NF-κB	nuclear factor κB
NK cells	natural killer cells
NMR	nuclear magnetic resonance
NO	nitric oxide
NPN	1-*N*-phenyl-napthylamine
NCLSS	National Committee of Laboratory Safety Standards

NCPF	National Collection of Pathogenic Fungi
NCTC	National Collection of Type Cultures
OaBac	*Ovine aries* bactenicin
OaDode	*Ovine aries* dodecapeptide
OD	optical density
PBSX	phosphate buffered saline plus magnesium chloride
PMAP	porcine myeloid antimicrobial peptide
PMN	polymorphonuclear leukocytes
RP-HPLC	reverse-phase high performance liquid chromatography
SBD	sheep β-defensin
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SMAP	sheep myeloid antimicrobial peptide
TEM	transmission electron microscopy
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TFE	2,2,2-trifluroethanol
TLRs	Toll-like receptors
TSB	tryptic-soy broth

LIST OF PUBLICATIONS

Most of the research presented in this thesis has been peer-reviewed and published in journals and/or presented at conferences. These publications are listed below. The full text of the journal articles are given in Appendix A5.

Journal Articles

Anderson RC, and Yu PL. (2003) Isolation and characterization of proline/arginine-rich cathelicidin peptides from ovine neutrophils. **Biochemical and Biophysical Research Communications** 312(4), 1139-1146.

Anderson RC, Wilkinson B, and Yu PL. (2004) Ovine antimicrobial peptides: new products from an age-old industry. **Australian Journal of Agricultural Research**, 55(1), 69-75.

Anderson RC, Hancock REW, and Yu PL. (2004) Antimicrobial activity and bacterial membrane interaction of ovine-derived cathelicidins. **Antimicrobial Agents and Chemotherapy**, 48(2), 673-676.

Anderson RC, Haverkamp R and Yu PL. (2004) Investigation of morphological changes to *S. aureus* induced by ovine-derived antimicrobial peptides using TEM and AFM. FEMS **Microbiology Letters**, 240(1), 105-110.

Anderson RC and Yu PL.(2005) Factors affecting the antimicrobial activity of ovine-derived cathelicidins against *E. coli* 0157:H7. **International Journal of Antimicrobial Agents**, 25(3), 205-210.

Anderson RC and Yu PL. Purification and characterisation of two protein fragments with antimicrobial activity from ovine blood, including part of the cathelicidin precursor. (waiting for Meat and Wool NZ approval to submit)

Anderson RC and Yu PL. Pilot-scale extraction and antimicrobial activity of crude extract from ovine neutrophils. (waiting for Meat and Wool NZ approval to submit)

Conference Proceedings

Anderson RC, Hancock REW and Yu PL (2003) Mechanism of action of ovine-derived antimicrobial peptides. **New Zealand Institute of Chemistry Conference**, 30th Nov-4th Dec 2003, Nelson, New Zealand.

Anderson RC, Wilkinson B and Yu PL (2002) Separation and activity of antimicrobial peptides from ovine blood. **American Society of Microbiology General Meeting**, 19-24th May, Salt Lake City, Utah, USA.

Anderson RC, Wilkinson B and Yu PL (2001) Purification of antimicrobial peptides from sheep's blood. **Proceedings of the Molecules for Life Conference**, 6-9th November 2001, Napier, New Zealand.

Yu PL and **Anderson RC** (2004) Ovine Antimicrobial peptides: How much do we know? **New Zealand Microbiological Society Annual Conference**. 17th - 19th November 2004, Palmerston North, New Zealand