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.

TABLE OF CONTENTS

CHAPTER 3
MATERIALS AND METHODS

CHAPTER 5

SPECTRUM OF ACTIVITY AND BACTERIAL MEMBRANE INTERACTIONS OF SYNTHETIC OVINE CATHELICIDINS

CHAPTER 6

MORPHOLOGY OF BACTERIAL CELLS TREATED WITH SYNTHETIC OVINE CATHELICIDINS

CHAPTER 7

FACTORS AFFECTING THE ANTIMICROBIAL ACTIVITY OF SYNTHETIC OVINE CATHELICIDINS AGAINST *E. COLI* O157:H7

APPENDIX A3

RAW DATA AND CALCULATIONS FROM EFFECT OF CONDITIONS STUDIES

APPENDIX A4

RAW DATA AND CALCULATIONS FROM PILOT-SCALE EXTRACTION STUDIES

APPENDIX A5

PEER-REVIEWED PUBLICATIONS

LIST OF FIGURES

LIST OF TABLES

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

CHAPTER 1
PROJECT INTRODUCTION

1.1 REASON FOR THE RESEARCH

This research project was carried out to investigate the properties of antimicrobial peptides that naturally occur in ovine blood cells. These antimicrobial peptides are of interest because they have the potential to be used in new high-value products for the New Zealand sheep industry. In New Zealand the meat industry is the second largest export earner, after the dairy industry (Statistics New Zealand, 2001). Of this, 45% of the earnings come from the sale of sheep meat (Benincasa *et al*, 2003), so the sheep industry is very important for the national economy.

Due to the large sheep industry in New Zealand, ovine blood is readily available. Each year approximately 25 million lambs and 5 million sheep are slaughtered nationwide (Benincasa *et al*, 2003). During the slaughtering process it is possible to recover 2.3 litres of blood per adult sheep and 1.1 litres of blood per lamb (Fernando, 1976). This means that approximately 40 million litres of ovine blood could be collected in New Zealand annually.

Currently, ovine blood is not utilised well by the New Zealand sheep industry. Ovine blood is either processed into low-value products, such as dried blood meal, which sells for approximately US$0.35/kg, or it is discarded as effluent (van Asch, 2001). It would be more beneficial to the sheep industry, and in turn the New Zealand economy as a whole, if this blood was further processed into high-value products.

The ovine blood could be collected during slaughter, separated and then further processed into a number of high-value products. Such products include blood serum, which is used in laboratories for growth of cell cultures, blood plasma proteins, including serum albumins, fibronectin, transferrin, antibodies, and trypsin, and red blood cell fractions including haemin and amino acids (Ockerman and Hansen, 1988).

As well as these products from blood plasma and red blood cells, the antimicrobial peptides could be extracted from the white blood cells and used in high-value products. These antimicrobial peptides have the potential to be used in a biopreserving solution for chilled lamb products. It is possible that this biopreserving solution could inhibit the growth of food spoilage organisms and increase shelf-life, and it could inhibit the growth of food-poisoning

organisms and ensure the safety, of chilled lamb products. The antimicrobial peptides also have the potential to be used in topical antiseptic creams. It is possible that such creams could be used to protect cuts and grazes from becoming infected, or as treatments for fungal infections such as athlete's foot.

1.2 PROJECT OBJECTIVES

The aim of the research presented in this thesis was to gain an understanding of the properties of antimicrobial peptides found in ovine blood neutrophils, so that their potential to be utilised in high-value products could be assessed. Although animal antimicrobial peptides have been studied by numerous research groups, few studies have focussed on ovine antimicrobial peptides, so relatively little is known about these peptides.

In order to learn more about ovine antimicrobial peptides, five project objectives were created. A literature review was carried out to establish what was already known about antimicrobial peptides in general, as well as ovine antimicrobial peptides specifically. This is presented in Chapter 2. From this, areas where knowledge was lacking were identified and the aims of this project were created accordingly.

The first objective of the research presented in this thesis was to purify and identify antimicrobial peptides from ovine blood. Prior to this research project, a crude extract had been produced from ovine white blood cells that had antimicrobial activity (Anderson and Yu, unpublished results). It was assumed that this activity was due to antimicrobial peptides because seven such peptides had been predicted from ovine cDNA; however, only one had been isolated from ovine blood.

The second objective of the research presented in this thesis was to determine the mechanisms of action of ovine antimicrobial peptides. The mechanisms of action of antimicrobial peptides are not fully understood; however, the mechanisms used seem to depend on the structural class of the peptide. Because the predicted ovine antimicrobial peptides were from more than one structural class, this research aimed to compare the interaction of different ovine antimicrobial peptides with bacterial membranes.

The third objective of the research presented in this thesis was to investigate the morphological changes to microbial cells induced by ovine antimicrobial peptides. Antimicrobial peptides cause different morphological changes to microbial cells depending on

their mechanism of action. Therefore, this research aimed to compare the morphological changes induced by different ovine antimicrobial peptides to further understand their mechanisms of action.

The fourth objective of the research presented in this thesis was to determine the effect of different environmental factors on the activity of ovine antimicrobial peptides. The activities of some antimicrobial peptides are inhibited by high salt concentrations, divalent cations and acidic pH values; whereas the activities of other peptides are not. The effects of such factors are important as they may limit the types of applications that the ovine antimicrobial peptides may be used in.

The fifth objective of the research presented in this thesis was to determine whether it is possible to produce an active antimicrobial extract on a scale larger than that used in the laboratory, using industrial-style equipment. Large-scale extractions of antimicrobial peptides have not been previously reported. If the peptides are to be used in commercial products, they need to be able to be isolated cost-effectively.

From the results of these five objectives it was hoped that enough information would be gained to decide whether product development to utilise the ovine antimicrobial peptides should be carried out. For this to occur, the antimicrobial peptides need to be robust and active in conditions that are likely in a product. As well as this, the peptides need to be present in the blood at concentrations high enough that the extraction is economical and the extraction process needs to be easily scaled-up to industrial-style operations.

CHAPTER 2
ANTIMICROBIAL PEPTIDES LITERATURE REVIEW

2.1 INTRODUCTION

The first objective of this literature review was to examine the characteristics of antimicrobial peptides. Antimicrobial peptides are produced by all forms of life to protect themselves against invading microorgansims. There are a number of structural classes of antimicrobial peptides that were investigated, including those produced by animals, plants and microbes.

The second objective of this literature review was to investigate the natural role of antimicrobial peptides within the immune system of animals. Firstly, an overview of the animal immune system, including both the innate and adaptive immune systems was carried out. Then, the function that antimicrobial peptides perform within these immune systems was examined.

The third objective of this literature review was to determine possible applications of antimicrobial peptides in order to show the reason for carrying out this research. Antimicrobial peptides are bioactive compounds that could be used in a number of areas including human health, animal health and food preservation. Firstly, the applications developed to date for antimicrobial peptides are reviewed. Then, possible applications for ovine blood antimicrobial peptides are proposed.

The fourth objective of this literature review was to investigate in detail the five areas relating to the objectives of this research project. These include purification of antimicrobial peptides from blood, mechanism of action of antimicrobial peptides, morphological changes to bacterial cells induced by antimicrobial peptides, effects of environmental conditions on activity of antimicrobial peptides and large-scale extraction of antimicrobial peptides from blood. The current knowledge in each of these areas is summarised and the techniques used to gather this knowledge are analysed.

The final objective of this review was to identify areas within these five target research fields where knowledge is lacking. From this, the objectives and hypotheses of the project were developed, with the aim of building on, and extending, the current knowledge in this field, specifically in relation to ovine blood antimicrobial peptides.

2.2 ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are small strings of amino acids, fewer than 100 residues long, that inhibit the growth of, and in some cases kill, microorganisms. These peptides are produced by all forms of life, including animals, plants and microorganisms, to protect themselves against microbial invasions. Antimicrobial peptides are part of the innate immune system of the host and they play a role in the first line of defence against microbial invasions.

The antimicrobial peptides produced by animals and plants have broad-spectrum activity. This is necessary because the peptides are required to protect the host from a wide range of microorganisms. The predominant function of antimicrobial peptides is to inhibit the growth of bacteria; however other organisms such as fungi (Selsted *et al*, 1985; Alcouloumre *et al*, 1993; Giacometti *et al*, 1999; Newman *et al*, 2000), protozoa (Aley *et al*, 1994), amoeba (Schuster and Jacob, 1992), viruses (Lehrer *et al*, 1985; Daher *et al*, 1986; Robinson *et al*, 1998; Yasin *et al*, 2000; Bastian and Schafer, 2001) and tumour cells (Sheu *et al*, 1985; Lichtenstein *et al*, 1986; Winder *et al*, 1998; Johnstone *et al*, 2000; Shin *et al*, 2000b) are also susceptible to some of these peptides.

In most cases, the antimicrobial peptides produced by microorganisms have a narrower spectrum of activity. This is necessary so that the host microbes themselves are not susceptible to the peptides. This means that the antimicrobial peptides produced by microbes target particular features of other microbes. The target microbes are usually those that compete with the host for an ecological niche.

Antimicrobial peptides can have a variety of different structures and are usually classified according to these structures. In the following subsections the structural classes of antimicrobial peptides produced by animals, plants and microbes are discussed in succession and the properties of each group of peptides are examined.

2.2.1 Animal antimicrobial peptides

Over the past few decades numerous animal antimicrobial peptides have been isolated and investigated. More than 800 animal antimicrobial peptide sequences are now stored in the antimicrobial peptides online database at http://www.bbcm.univ.trieste.it/~tossi/pag2.htm.

There are four main structural classes of antimicrobial peptides produced by animals. Examples of animal antimicrobial peptides from each structural class are given in Figure 2.1.

Although the structures of antimicrobial peptides vary considerably, they have a number of common features. They all have separate charged and hydrophobic regions, and they are all cationic, with a positive charge of at least two. These properties help them to interact with bacterial membranes.

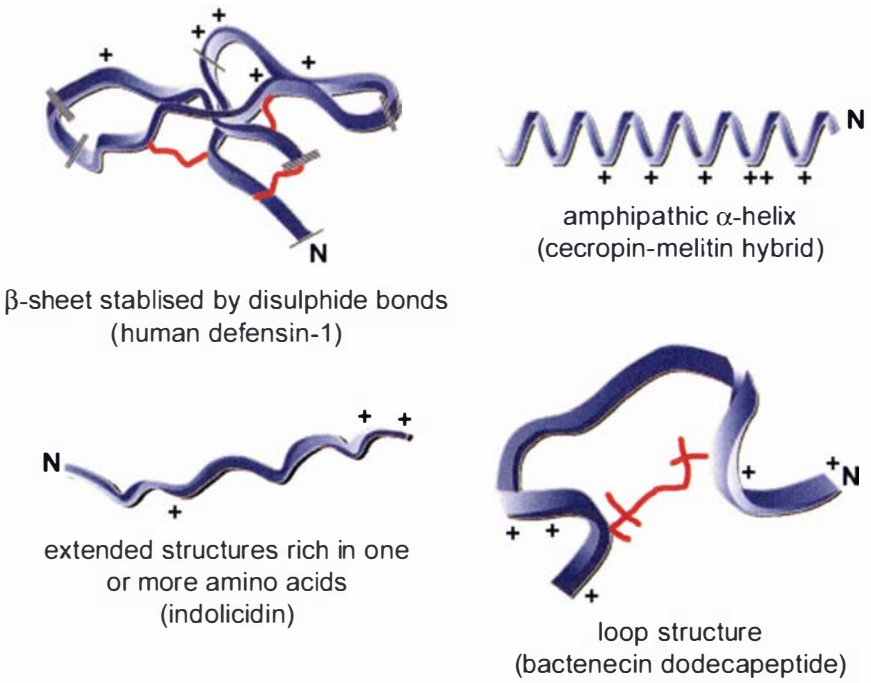

Figure 2.1 - Examples of the four structural classes of cationic antimicrobial peptides. The positive residues are marked with a '+', the N-terminus is marked with an 'N' and the disulphide bonds are shown in red. This figure is adapted from that given by Hancock, 1997.

The first structural class of animal antimicrobial peptides are predominately β-sheets. These peptides have an even number of cysteine residues and are stabilised by disulphide bonds. Members of this structural class include the defensins produced by mammals and birds, the insect defensins, big defensin produced by horseshoe crabs, and penaeidins produced by shrimp, which have three disulphide bonds; and the protegrins produced by pigs, the tachyplesins produced by horseshoe crabs, and androctonin produced by scorpions, which have two disulphide bonds (Dimarcq *et al*, 1998).

The second structural class of animal antimicrobial peptides are amphipathic α-helices. Members of this structural class include the myeloid antimicrobial peptides (MAP) produced by mammals, the cecropins produced by insects (Otvos, 2000), and the bombinins, bombinin-like peptides, magainins, brevinins, dermaseptins and caerins produced by amphibians (Simmaco *et al*, 1998).

The third structural class of animal antimicrobial peptides are extended linear structures. These peptides are rich in one or more amino acid. Members of this structural class include the proline/arginine-rich Bac peptides and the tryptophan-rich indolicidin produced by mammals and the proline-rich, short peptides produced by insects (Otvos, 2002).

The final structural class of animal antimicrobial peptides are hairpin structures. These peptides contain one disulphide bond to hold them in their loop shape. Members of this structural family include the bactenecin dodecapeptides produced by mammals and thanatin produced by insects (Otvos, 2000)

Due to the large number of families of animal antimicrobial peptides, only the families of peptides produced by mammals are reviewed in detail. The antimicrobial peptides produced by mammals fall into two families, the defensins and the cathelicidins. The characteristics of these families are discussed below. The characteristics of the individual peptides that have been purified from animal blood are discussed later in Section 2.5.2.

The first family of mammalian antimicrobial peptides are the defensins. Defensins are also produced by other higher animals such as birds. These peptides are rich in arginine residues and contain six cysteine residues that form three disulphide bonds. These disulphide bonds stabilise the peptides which fold into a three-stranded, antiparallel β-sheet structure. The defensins are divided into groups according to the position and connecting pattern of the cysteine residues. Examples of defensins from each of the groups are given in Figure 2.2

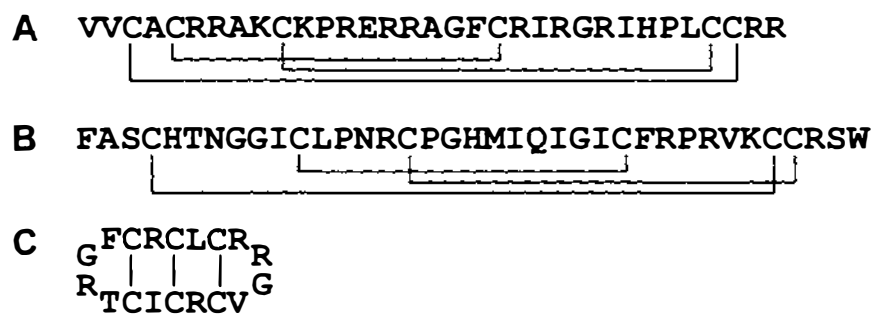

Figure 2.2 - Examples of the three groups of defensins. (A) is rabbit α-defensin NP-1, (B) is bovine β-defensin BNBD-1, and (C) is monkey θ-defensin RTD-1. The lines connecting the cysteine residues show the disulphide-bond linking patterns.

The first group of mammalian defensins to be isolated were the α-defensins (Selsted *et al*, 1985). These peptides are 29-35 residues long, and contain six cysteine residues that are linked in a 1-6, 2-4, and 3-5 pattern (Selsted and Harwig, 1989). The α-defensins are

commonly found in the neutrophils of mammals, where they are synthesised then stored in the specific granules. α-defensins have been identified in the neutrophils of humans (Wilde *et al*, 1989), monkeys (Tang *et al*, 1999a), rabbits (Fuse *et al*, 1993), rats (Eisenhauer *et al*, 1989) , guinea pigs (Selsted and Harwig, 1987; Yamashita and Saito, 1989) and hamster (Mak *et al*, 1996). A sub-group of α-defensins has also been isolated from the gastrointestinal tract of mice (Ouellette and Lualdi, 1990; Eisenhauer *et al*, 1992; Ouellette *et al*, 1992; Ouellette *et al*, 1999). These intestinal peptides are also known as cryptdins. Another sub-group of α-defensins has been identified in the kidney of rabbits (Bateman *et al*, 1996; Wu *et al*, 1998).

The second group of mammalian defensins, the β-defensins, are slightly bigger than the α-defensins, with 38-42 residues, and have the cross-linking pattern 1-5, 2-4, and 3-6 (Selsted *et al*, 1993). β-defensins have N-terminal α-helices that allow them to form dimers. These dimers then group together to form octamers (Hoover *et al*, 2000). The β-defensins are predominately found in the epithelia of the gastrointestinal, respiratory and urogenital tracts of mammals. β-defensins have been identified in these locations in humans, (Diamond *et al*, 1991; Jones and Bevins, 1993; Diamond *et al*, 1993; Schnapp *et al*, 1998; Krisanaprakornkit *et al*, 1998; Mathews *et al*, 1999; Bonass *et al*, 1999; Jia *et al*, 2001), chimpanzees (Duits *et al*, 2000), monkeys (Bals *et al*, 2001), mice (Huttner *et al*, 1997; Jia *et al*, 2000), rats (Jia *et al*, 1999; Li *et al*, 2001), sheep (Huttner *et al*, 1998), goats (Zhao *et al*, 1999), horses (Davis *et al*, 2004) and chickens (Lynn *et al*, 2004). However, numerous β-defensins have also been found in the neutrophils of cattle (Selsted *et al*, 1993), chickens (Evans *et al*, 1994; Brockus *et al*, 1998), turkey (Evans *et al*, 1994; Brockus *et al*, 1998) and ostrich (Yu *et al*, 2001).

There is a third group of defensins, the θ-defensins, which have been isolated from *Rhesus macaque* monkeys. The first θ-defensin, RTD-1, is biosynthesised as two abbreviated α-defensins, each 9 amino acids long and these peptides are stabilised by three disulphide bonds to form a cyclic peptide (Tang *et al*, 1999b; Trabi *et al*, 2001). The two 9 amino acid peptides that make up RTD-1 have been termed RTD-1a and RTD-1b. Two other θ-defensins, RTD-2 and RTD-3, have also been isolated. RTD-2 and RTD-3 are homodimers of RTD-1b and RTD-1a respectively (Tran *et al*, 2002). However, RTD-1 is more abundant in monkey neutrophils. The ratio of RTD-1, 2, and 3 is 29:1:2.

Defensins are synthesised as prepropeptides. The prepiece is a 19 amino acid endoplasmic signal sequence, which is cleaved during translation. The propiece is a 45 residue, anionic

sequence and is not removed until the mature peptide is required (Nakazato *et al*, 1995). Studies have shown that the anionic propiece inhibits the activity of the defensins to protect host cells from their cytotoxicity (Valore *et al*, 1996). A segment of this propiece is also essential for the subcellular trafficking and sorting of the defensins (Liu and Ganz, 1995).

The second family of antimicrobial peptides found in mammals is the cathelicidins. Originally, cathelicidins were thought to be unique to mammals, but recently they have been identified in chickens (Lynn *et al*, 2004) and hagfish (Uzzell *et al*, 2003), which indicates they are present in other types of animals.

Cathelicidins are synthesised as prepropeptides. The structure of these prepropetpides is illustrated in Figure 2.3. The N-terminal preproregion is highly conserved between species (Storici *et al*, 1992). It is very similar to a porcine cysteine-protease inhibitor, cathelin, hence the name cathelicidins (Zanetti *et al*, 1995). The signal peptide is 29 and the proregion is 101 amino acids long.

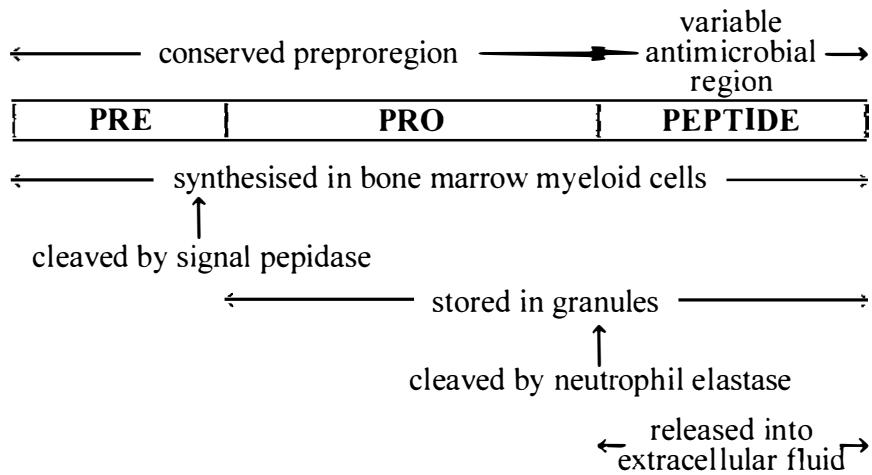

Figure 2.3 - Schematic diagram of a cathelicidin. This figure is adapted from that given by Zanetti *et al*, 1990.

In contrast to the preproregions, the C-terminal regions of cathelicidins are highly variable. They can have structures that fall into any of the four different structural classes described previously. A single species of animal can produce numerous cathelicidin peptides that are highly variable in sequence and structure, yet some cathelicidin peptides are conserved between species. α-Helical cathelicidins have been identified in humans (Larrick *et al*, 1995; Agerberth *et al*, 1995; Cowland *et al*, 1995; Bals *et al*, 1998; Murakami *et al*, 2002), monkeys (Zhao *et al*, 2001; Bals *et al*, 2001), mice (Larrick *et al*, 1994; Popsueva *et al*, 1996; Gallo *et al*, 1997), rabbits (Larrick *et al*, 1991; Hirata *et al*, 1994; Tossi *et al*, 1994), guinea pigs

(Nagaoka *et al*, 1997), cattle (Skerlavaj *et al*, 1996; Scocchi *et al*, 1997), pigs (Storici *et al*, 1994; Shi *et al*, 1994; Zanetti *et al*, 1994; Tossi *et al*, 1995), sheep (Bagella *et al*, 1995; Mahoney *et al*, 1995; Huttner *et al*, 1998) and horses (Scocchi *et al*, 1999). Linear cathelicidins rich in one or more animo acids have been found in cattle (Gennaro *et al*, 1989; Frank *et al*, 1990; Selsted *et al*, 1992; Scocchi *et al*, 1998), pigs (Agerberth *et al*, 1991; Harwig *et al*, 1995; Zhao *et al*, 1995b), sheep (Bagella *et al*, 1995; Huttner *et al*, 1998), and goats (Shamova *et al*, 1999). Cathelicidins containing two disulphide bonds have been identified in pigs (Storici and Zanetti, 1993; Zhao *et al*, 1994; Steinberg *et al*, 1997), while cathelicidins containing one disulphide bond have been identified in cattle (Romeo *et al*, 1988) and sheep (Bagella *et al*, 1995; Huttner *et al*, 1998).

Cathelicidins are located in a variety of cells within the host. The majority of cathelicidins are synthesised in maturing myeloid cells in the bone marrow as prepropeptides. The preregion is removed by signal peptidase, and then the majority of the propeptides are stored in the neutrophil granules (Zanetti *et al*, 1990). Several cathelicidins have also been found to be expressed in other leukocytes such as monocytes, NK cells, T lymphocytes and B lymphocytes, and in epithelial cells of the skin (Frohm *et al*, 1997; Murakami *et al*, 2002), gastrointestinal tract, respiratory tract (Bals *et al*, 1998) and urogenital tract (Malm *et al*, 2000).

Cathelicidin peptides are not active until the propiece is proteolytically removed (Scocchi *et al*, 1992). In the case of two bovine cathelicidins that were studied, this cleavage is carried out by neutrophil elastase during degranulation into the phagocytic vacuole or extracellular fluid (Zanetti *et al*, 1991; Del Sal *et al*, 1992; Zanetti *et al*, 1993). It has also been shown that the inhibition of neutrophil elastase prevented cathelicidin activation and impaired clearance of bacteria from a porcine model wound chamber (Cole *et al*, 2001). In contrast, the human cathelicidin LL-37 is cleaved by proteinase 3 (Sorensen *et al*, 2001), and not neutrophil elastase, which shows that cathelicidin processing is governed by different enzymes in different species.

Cathelicidins are products of individual genes that code for the corresponding prepropeptide. The structures of the cathelicidin genes are conserved between species. They contain four exons as shown in Figure 2.4. The first three exons code for the cathelin-like prosequence, and the fourth exon codes for the active peptide. In animal species with more than one

cathelicidin, the genes are usually arranged in clusters (Zhao *et al*, 1995a; Scocchi *et al*, 1997).

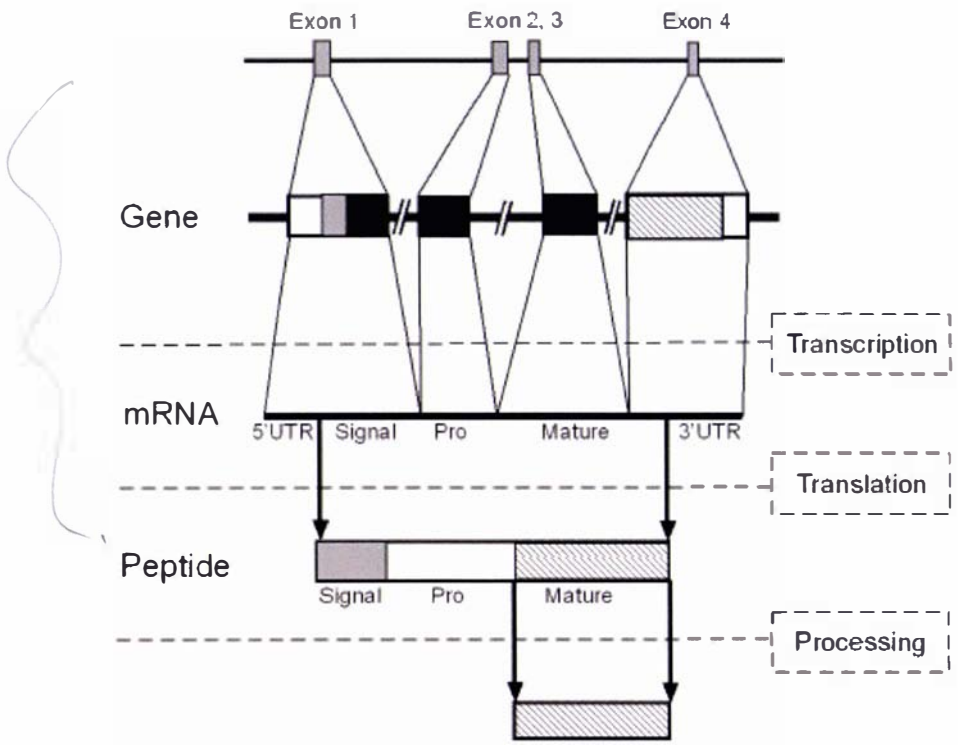

Figure 2.4 - Schematic diagram of the gene for the human cathelicidin LL-37. This figure is adapted from that given by Bals and Wilson, 2003.

2.2.2 Plant antimicrobial peptides

Like animals, plants produce numerous antimicrobial peptides to protect themselves from invading microbes. The plant antimicrobial peptides are also classified into families according to their structures. The majority of these structural families, including the thionins, plant defensins, lipid transfer proteins, heven-type peptides and knottin-type peptides, contain six or eight cysteine residues that form three or four disulphide bonds. The properties of each family of plant antimicrobial peptides are summarised below.

The first family of antimicrobial peptides produced by plants are the thionins, which range from 45 to 47 amino acids long. Thionins are separated into two subgroups depending on whether they have six or eight cysteine residues (Broekaert *et al*, 1997). The secondary and tertiary structures of these peptides are given in Figure 2.5. There is a third group of thionins which appears to be a truncated version of the eight cysteine thionins, which contain only six cysteine residues.

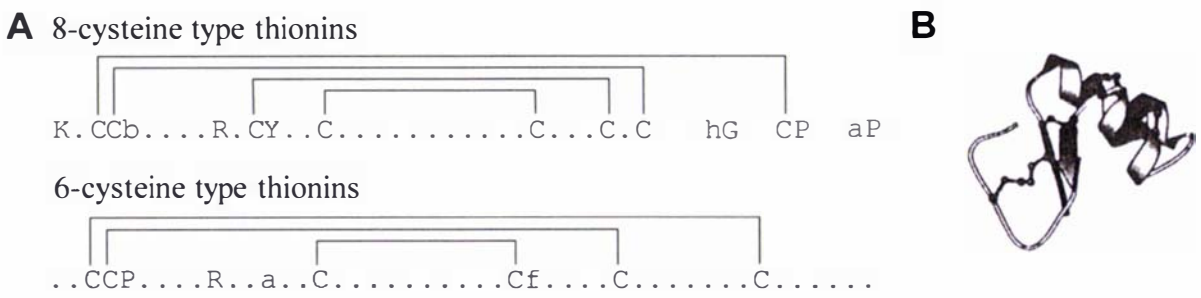

Figure 2.5 - Structure of thionins. 'A' shows the secondary structures of thionins with six and eight cysteine residues. The conserved amino acids are given in capital letters and the lines show the disulphide bonds. 'a' represents aromatic residues, 'b' represents basic residues and 'h' represents hydroxy residues. 'B' shows the tertiary structure of thionins. The helices represent α-helices and the arrows represent β-strands. This image was adapted from that given by Broekaert *et al*, 1997.

Thionins have been isolated from a wide range of plants including both monocots and dicots. They are expressed in a range of tissues, for example, in barley, thionins have been found in the endosperm of seed, in leaves and in roots (Broekaert *et al*, 1997). The thionin expression in the different organs is encoded by different genes displaying organ-specific expression. Thionins are synthesised by membrane bound polysomes as preproproteins with the mature peptide being located between the N-terminal signal domain and the C-terminal prodomain (Garcia-Olmedo *et al*, 1998).

Thionins inhibit the growth of both Gram-positive and Gram-negative bacteria as well as numerous phytopathogenic fungi (Broekaert *et al*, 1997). The concentration of thionins required to reduce the growth of microorganisms ranges between 1 and 15μg/mL. Thionins are also toxic to insects and mammals when injected into their body fluids, but not when taken orally.

The second family of plant antimicrobial peptides are the plant defensins. Plant defensins are similar in size to thionins, 45 to 54 amino acids, and like some thionins they contain eight cysteine residues that form four disulphide bonds (Broekaert *et al*, 1997). The secondary and tertiary structures of these peptides are given in Figure 2.6.

Plant defensins have been isolated from numerous plant species (Broekaert *et al*, 1997). The tissues in which they occur include leaves, flower organs, pods and seeds. Most plant defensins are synthesised with a signal peptide but without a propeptide; however, in some cases propeptides are present at the C-terminus (Garcia-Olmedo *et al*, 1998).

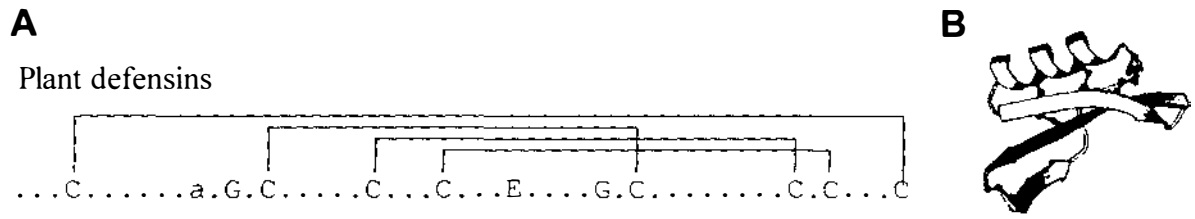

Figure 2.6 - Structure of plant defensins. 'A' shows the secondary structures of plant defensins. The conserved amino acids are given in capital letters and the lines show the disulphide bonds. 'a' represents aromatic residues. 'B' shows the tertiary structure of plant defensins. The helices represent α-helices and the arrows represent β-strands. This image was adapted from that given by Broekaert *et al*, 1997.

Plant defensins are predominately active against fungi, and in most cases have only limited activity against bacteria (Broekaert *et al*, 1997). According to the way the plant defensins act on fungi they are separated into two groups. The morphogenic plant defensins cause reduced hyphal elongation with a concomitant increase in hyphal branching, whereas the nonmorphogenic plant defensins slow down hyphal elongation but do not induce marked morphological distortions (Broekaert *et al*, 1997).

The third group of plant antimicrobial peptides are the lipid transfer proteins (LTPs). These peptides are longer than those already discussed as they consist of 90-93 amino acids (Broekaert *et al*, 1997). LTPs contain eight cysteine residues that form four disulphide bonds. The secondary and tertiary structures of these peptides are given in Figure 2.7.

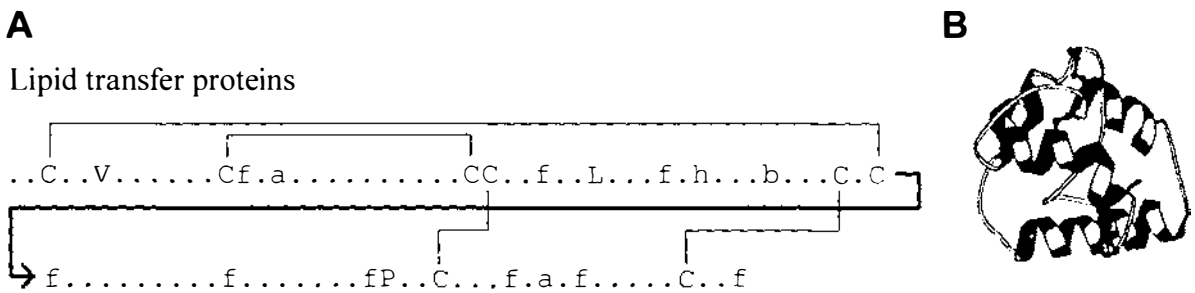

Figure 2.7 - Structure of lipid transfer proteins. 'A' shows the secondary structures of lipid transfer proteins. The conserved amino acids are given in capital letters and the lines show the disulphide bonds. 'a' represents aromatic residues, 'b' represents basic residues, 'f' represents hydrophobic residues and 'h' represents hydroxy residues. 'B' shows the tertiary structure of lipid transfer proteins. The helices represent α-helices. This image was adapted from that given by Broekaert *et al*, 1997.

Like the thionins and plant defensins, LTPs have been found in numerous plant species in a variety of tissues (Broekaert *et al*, 1997). These include embryos, cotyledons, leaves, stems and flower organs. Like the defensins the majority of the LTPs are synthesised with signal domains, but only a few contain a C-terminal propeptide domain.

LTPs were originally detected on a bioassay that measured the transfer of phospholipids, hence the name (Broekaert *et al*, 1997). However, since then it has become apparent that not all LTPs have this property. The antimicrobial activity of LTPs also varies considerably between peptides.

The fourth group of plant antimicrobial peptides are the hevein and knottin-type peptides. Hevein-type peptides are similar to the chitin-binding protein produced by rubber trees, called hevein, and knottin-type peptides are similar to the knottin peptides that fold into a knot-like structure (Broekaert *et al*, 1997). Both types of peptides have the same cysteine motif and cysteine connective pattern as shown in Figure 2.8; however, hevein-type peptides often contain eight cysteine residues compared to the six cysteine residues found in knottin-type peptides.

Hevein-type peptides are synthesised with an N-terminal signal peptide and a C-terminal propeptide; whereas knottin-type peptides do not have propeptides (Broekaert *et al*, 1997). Both hevein- and knottin-type peptides have similar antimicrobial activity. They are active against Gram-positive bacteria and fungi.

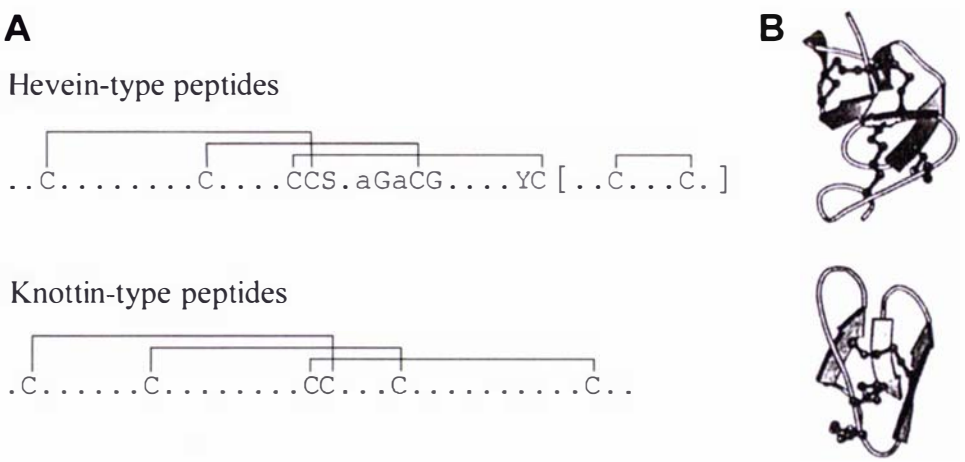

Figure 2.8 - Structure of hevein- and knottin-type peptides. 'A' shows the secondary structures of hevein- and knottin-type peptides. The conserved amino acids are given in capital letters and the lines show the disulphide bonds. 'a' represents aromatic residues. The brackets surrounded the C-terminus that is only present in some peptides. 'B' shows the tertiary structure of hevein- and knottin-type peptides. The arrows represent β-strands. This image was adapted from that given by Broekaert *et al*, 1997.

There are other families of plant antimicrobial peptides, including the two families of four-cysteine peptides and the twelve-cysteine peptides. These families are not as well studied as those previously discussed.

The first family of four-cysteine peptides contains only one member that is currently known (Garcia-Olmedo *et al*, 1998). This peptide is called MBP-1. MBP-1 is 33 residues long and its four cysteine residues are organised in two CXXXC segments. MBP-1 is active against numerous fungi and at least one Gram-positive and one Gram-negative bacteria (Broekaert *et al*, 1997).

The second family of four-cysteine peptides are called Ib-AMPs because they come from the seeds of *Impatients balsamina* (Garcia-Graells *et al*, 1999). The four members in this family are all processed from a single multi-peptide precursor (Broekaert *et al*, 1997). These peptides are active against Gram-positive bacteria and fungi.

The final family of plant antimicrobial peptides are called snakins. Currently, there is only one known peptide in this family (Garcia-Graells *et al*, 1999). Snakin-1 was isolated from the tuber of potatoes and it is 63 residues long and contains twelve cysteine residues. This peptide is active against Gram-negative and Gram-positive bacteria and fungi.

2.2.3 Microbial antimicrobial peptides

As well as animals and plant, microorganisms produce antimicrobial peptides. It is thought that 99% of bacteria produce antimicrobial peptides; however, relatively few of these have been isolated to date. The majority of antimicrobial peptides produced by bacteria have a narrow spectrum of activity and are toxic only to bacteria closely related to the producing strain. This allows bacterial strains to compete with similar strains for ecological niches. Some antimicrobial peptides produced by bacteria have broad-spectrum activity. The most well studied of these is nisin, which is used in the food industry as a preservative.

The antimicrobial peptides produced by bacteria are commonly called bacteriocins. Like the animal and plant antimicrobial peptides, bacteriocins are classified into structural families. The properties of each family of bacteriocins are summarised below.

The first class of bacteriocins contains unusual post-translationally modified amino acids, such as dehydroalanine, dehydrobutirine, lanthionine or β-methyl-lanthionine (Oscartz and Pisabarro, 2001). This class of bacteriocins is produced by Gram-positive, lactic acid bacteria and are often called lantibiotics.

Class I bacteriocins are divided into two groups depending on their structural features and mechanism of action (Oscartz and Pisabarro, 2001). Class Ia bacteriocins contain 21 to 38

amino acids and form screw-shaped, amphipathic structures. These peptides kill by depolarising the cytoplasmic membrane of the target cell. Class Ib bacteriocins contain fewer than 19 amino acids that form a globular structure. These bacteriocins function through enzyme inhibition (Riley and Wertz, 2002). Some examples of class Ia and class Ib bacteriocins are given in Figure 2.9.

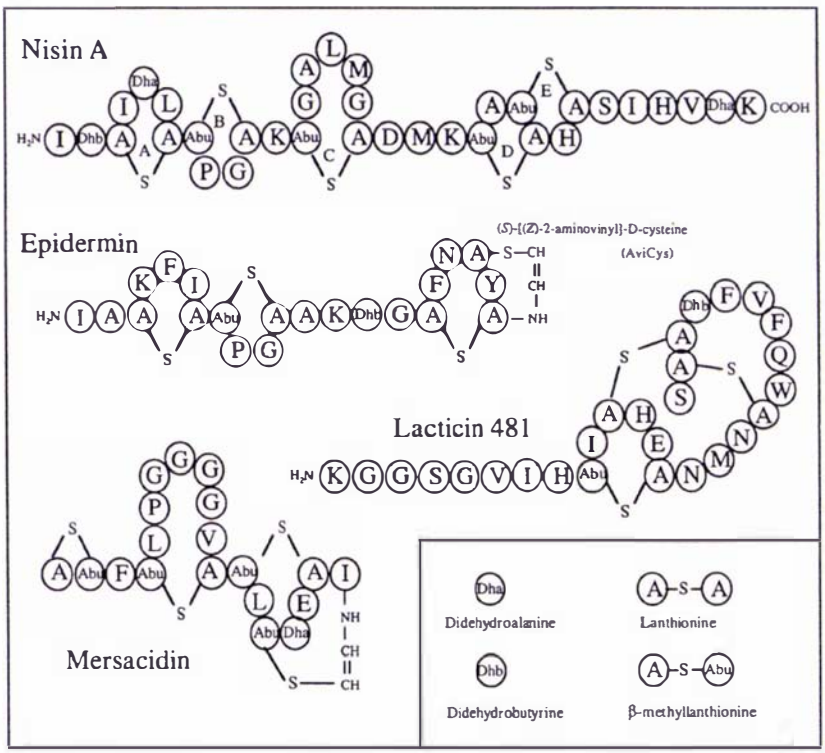

Figure 2.9 - Structures of four Class I bacteriocins. Nisin A, epidermin and lacticin 481 are class Ia bacteriocins. Mersacidin is a class Ib bacteriocin. This image was taken from McAuliffe *et al*, 2001.

The production of lantibiotics is a complicated process (McAuliffe *et al*, 2001). Firstly, the prelantibiotics are produced on the ribosome. These prepeptides then undergo extensive post-translational modification, including dehydration and cross-linking reactions. Finally, the leader peptide is cleaved to produce the active molecule, which is then secreted. For this reason, lantibiotics require a large number of genes. For example, the most well studied lantibiotic, nisin, has a gene cluster that includes genes for the prepeptide, enzymes for modifying the amino acids, cleavage of the leader peptide, secretion, immunity and regulation of expression (Riley and Wertz, 2002).

The second class of bacteriocins contain no modified amino acids, are heat-stable and are smaller than 10kDa (Oscartz and Pisabarro, 2001). Like Class I bacteriocins, the peptides are

produced by Gram-positive, lactic acid bacteria. The members of this class are divided into four groups.

The first group of class II bacteriocins, called class IIa bacteriocins, contain a concensus sequence of YGNGV at their N-terminus and are all active against Listeria (McAuliffe *et al*, 2001). These peptides also have sequence similarities in their C-termini (Ennahar *et al*, 2000). Class IIa bacteriocins are between 37 and 58 residues long and they act through formation of pores in the cytoplasmic membrane (Nes and Holo, 2000).

The second group of class II bacteriocins, called class IIb bacteriocins, are also pore forming complexes; however; they require two peptides for their activity (Oscartz and Pisabarro, 2001). In some cases the two peptides are active individually but are synergistic when acting together, in other cases both peptides are necessary for activity.

The final group of class II bacteriocins, called class IIc bacteriocins, includes all class II bacteriocins that do not fall into the first two groups (Oscartz and Pisabarro, 2001). This includes bacteriocins with one or two cysteine residues and bacteriocins without any cysteine residues.

As well as the class I and II bacteriocins there are two other classes of bacteriocins produced by Gram-positive bacteria. However, these classes have not been studied in as much detail. Class III bacteriocins are heat-labile proteins with masses higher than 30kDa (Oscartz and Pisabarro, 2001). Class IV bacteriocins are glycoproteins and lipoproteins that require non-protein moieties for their activity (Oscartz and Pisabarro, 2001).

Like Gram-positive bacteria, Gram-negative bacteria produce bacteriocins. The most well studied are the bacteriocins produced by the *Enterobacteriaceae* family (Oscartz and Pisabarro, 2001). These peptides are classified according to their sizes. Colicins are larger that 10kDa and microcins are smaller than 10kDa. Colicins have a narrow spectrum of activity because their activity is mediated by interaction with specific membrane receptors.

The Archaea produce a distinct family of antimicrobial peptides. These are known as archaeocins. The only characterised family of these peptides is the halocins which are produced by halobacteria. These peptides are extremely hardy. They can be desalted, boiled, subjected to organic solvents and stored at 4°C for extended periods without detrimental effects on their activity.

2.3 THE ANIMAL IMMUNE SYSTEM

Antimicrobial peptides are one component of the very complex immune system of animals. The immune system is a collection of cells, tissues and molecules that have the physiological functions of preventing new infections and eradicating established infections. The host defence system uses a number of mechanisms to protect itself from invading microbes. These mechanisms can be split into those that are part of innate immunity and those that are part of adaptive immunity as illustrated in Figure 2.10.

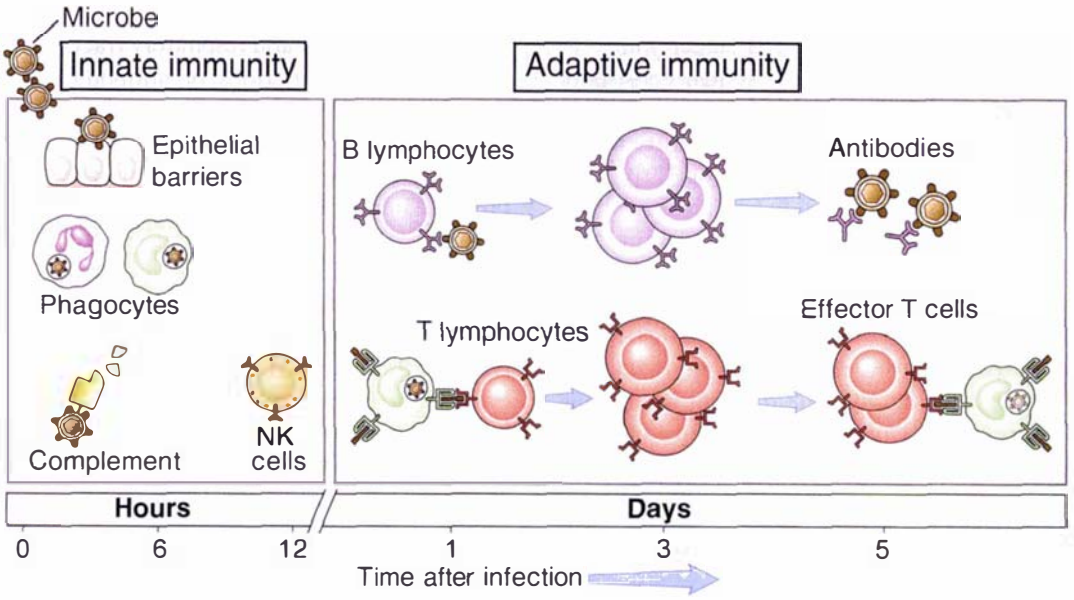

Figure 2.10 - Schematic diagram showing the principle mechanisms of innate and adaptive immunity. This image was taken from Abbas and Lichtman, 2004.

The innate immune system, which is also called the natural or native immune system, is always present in healthy individuals. It responds immediately to invading organisms and mediates the initial protection against infections by blocking entry of microbes and rapidly eliminating microbes that do enter the host tissues. The components of the innate immune system are generic and do not change to match a varying microbial assault.

In contrast, the adaptive immune system, which is also called the specific or acquired immune system, is not always present; instead it is stimulated by the presence of microbes in the host tissues. This system is much slower than the innate immune system to respond to infections; however, its mechanisms are more effective so it is required when the innate immune system is overwhelmed. The adaptive immune system is stimulated by the microbes that invade the

host and it adapts to the presence of the particular invaders. Adaptive immunity is relatively new in evolutionary terms and is present only in vertebrates.

The following sections firstly outline the components and mechanisms of the two immune systems; the innate and the adaptive immune systems. Then the role of animal antimicrobial peptides within these defence systems is examined.

2.3.1 Innate immunity

The innate immune system is that which the host is born with and which is always present and available at very short notice to protect the host from challenges from foreign invaders. There are a number of components that make up the innate immune system including epithelial barriers, phagocytes, natural killer (NK) cells and the complement system. These components play different but complementary roles in blocking the entry of microbes and eliminating microbes that enter the tissues of the host.

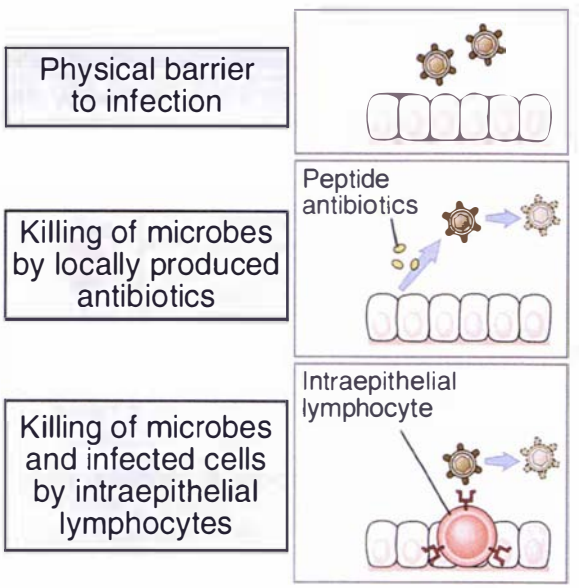

Figure 2.11 - Functions of the epithelia in innate immunity. This image was taken from Abbas and Lichtman, 2004.

The first component in the innate immune system to come in contact with invading organisms is the epithelial barrier. There are three main interfaces between the body and the external environment, which are the skin, the gastrointestinal tract and the respiratory tract. To prevent microbes entering the host via these interfaces, they are lined with epithelial cells. These epithelial cells protect the host in three ways, which are illustrated in Figure 2.11. Firstly, the epithelial cells form a physical barrier to exclude microbes. Secondly, they

produce and excrete peptide antibiotics to kill microbes before they enter host cells. Finally, they contain intraepithelial lymphocytes that kill microbes on contact.

The second component of the innate immune system is the phagocytes. Phagocytes are cells responsible for detecting and killing extracellular invading microbes. Phagocytes are recruited to the sites of infection where they recognise and then ingest microbes for intracellular killing. The phagocytosis and intracellular killing processes are illustrated in Figure 2.12.

There are two types of circulatory phagocytes, neutrophils and monocytes, which are cells of blood. Neutrophils, which are also called polymorphonuclear leukocytes (PMN), are the most abundant leukocytes in the blood. In response to infection the production of neutrophils in the bone marrow rises dramatically to increase their numbers from 4,000-10,000 to 20,000 per mL of blood. Monocytes are less abundant than neutrophils and have a concentration of 500-1,000 per mL of blood. Monocytes can also enter the extravascular tissues and differentiate into macrophages. Resident macrophages are found in the connective tissues and in every organ in the body.

Neutrophils and macrophages recognise microbes in the blood and extravascular tissues by surface receptors that are specific for microbial products. There are several different types of receptors which are specific for different structures or patterns that are frequently found on microbial molecules. Toll-like receptors (TLRs) are a group of receptors that are specific for different microbial components. Signals generated by engagement of TLRs activate a transcription factor called nuclear factor κB (NF-κB), which stimulates the production of cytokines, enzymes and other proteins involved in antimicrobial functions. Neutrophils and macrophages also express receptors that recognise other microbial structures such as mannose receptors, integrins (mainly one called Mac-1) and scavenger receptors.

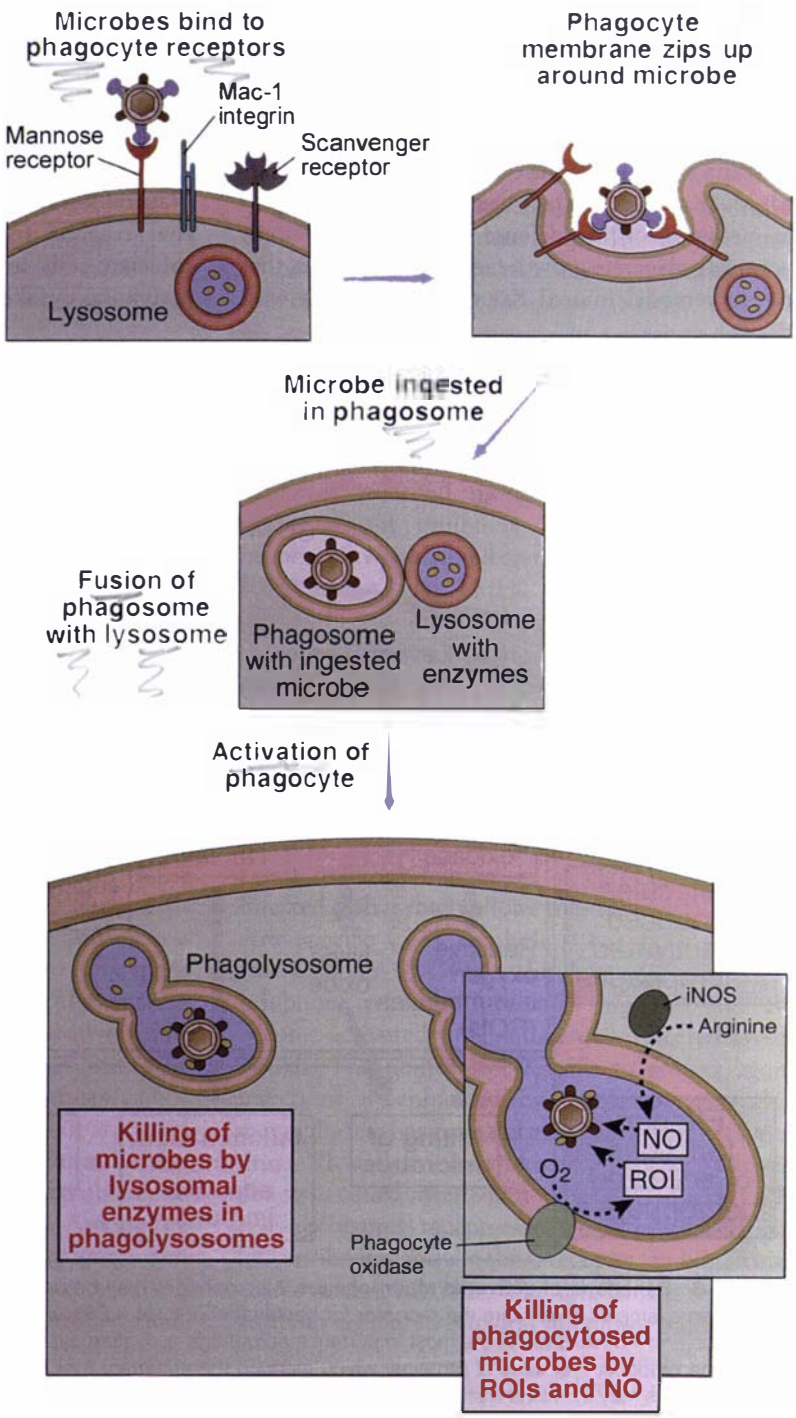

Figure 2.12 - Mechanism of phagocytosis and intracellular killing of microbes. NO is nitric oxide and ROI is reactive oxygen intermediate. This image was taken from Abbas and Lichtman, 2004.

Once the microbes are recognised by the phagocytes, phagocytosis occurs. This process begins with the phagocyte extending its plasma membrane around the recognised microbe. The membrane then closes up and pinches off, which leaves the microbe internalised in a membrane bound vesicle called a phagosome.

Once the microbes are inside the phagocytes, they are killed. The phagosomes fuse with lysosomes to form phagolysosomes. Lysosomes contain numerous enzymes that mediate the killing of microbes, including phagocyte oxidase, which converts molecular oxygen to superoxide anion and free radicals, inducible nitric oxide synthase catalyses, which mediates the conversion of arginine to nitric oxide (NO), and lysosomal proteases, which break down microbial proteins.

The third component of the innate immune system is the natural killer (NK) cells. NK cells are responsible for killing intracellular microorganisms. They do this by destroying infected host cells and by producing interferon-γ (IFN-γ), which is a cytokine that activates macrophages containing phagocytosed microbes. The functions of NK-cells are illustrated in Figure 2.13.

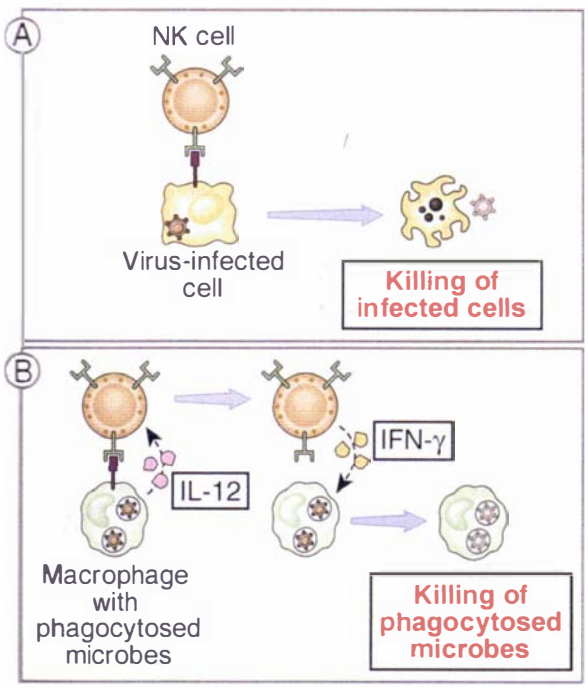

Figure 2.13 - Functions of the natural killer (NK) cells. A) NK cells kill infected host cells and B) NK cells activate macrophages to kill phagocytosed microbes. IL-12 in interleukin-12 and IFN-γ is interferon-γ. This image was taken from Abbas and Lichtman, 2004.

One role of NK cells is to recognise any host cells infected with microorganisms. The mechanism of recognition is not fully understood. However, it is known that NK cells express various receptors for molecules on host cells and that some of these receptors activate NK cells and some inhibit them. The activating receptors recognise molecules commonly expressed on infected host cells. Once the NK cells are activated they discharge proteins

from their cytoplasmic granules into the infected cells. These proteins induce apoptosis in the infected cells.

The other role of NK cells is to activate macrophages containing phagocytosed microbes. They do this by producing IFN-γ, which stimulates macrophages to become more effective at killing phagocytosed microbes. The production of IFN-γ is induced by interleukin (IL)-12 which is produced by the macrophages.

The final component of the innate immune system is the complement system. This is a collection of circulating and membrane-associated proteins that are important in defence against microbes. The complement activation involves the sequential activation of various proteolytic enzymes. This cascade can be activated by three different pathways as illustrated in Figure 2.14. The alternative and lectin pathways are part of the innate immune system but the classical pathway is part of the adaptive immune system.

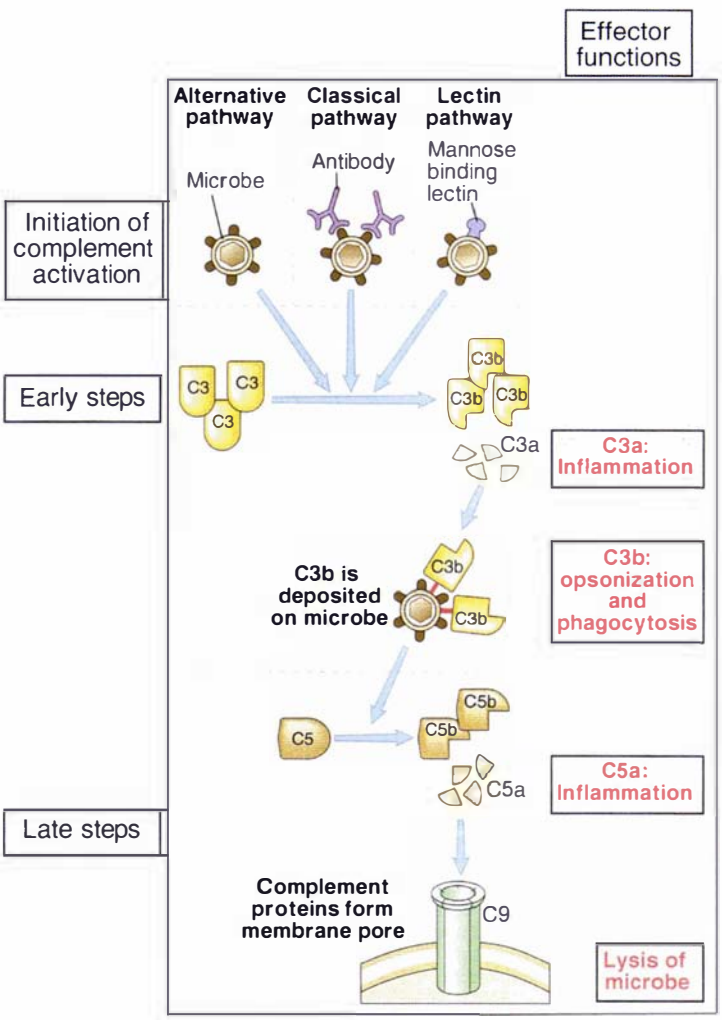

Figure 2.14 - Pathways of activation of the complement system. This image was taken from Abbas and Lichtman, 2004.

The complement system has three functions in host defence. The central component of the complement system is a plasma protein called C3, which is cleaved by the enzymes generated in the early steps of the cascade. The major fragment of C3, called C3b is responsible for two of the functions of the complement system. Firstly, it coats microbes to promote their binding to phagocytes. Secondly, some of its breakdown products are chemoattractants for neutrophils and macrophages, and promote inflammation. The third function of the complement system is to form a polymeric protein complex that inserts into the microbial cell membrane to form pores. These pores allow the influx of water and ions, which leads to the death of the microbial cell.

2.3.2 Adaptive immunity

The adaptive immune system is much slower to respond than the innate immune system; however, it is more powerful at eliminating microbes from the host. There are two types of adaptive immunity, humoral and cell-mediated immunity, which are designed to provide defence against extracellular and intracellular microbes respectively. The functions of these systems are outlined in Figure 2.15.

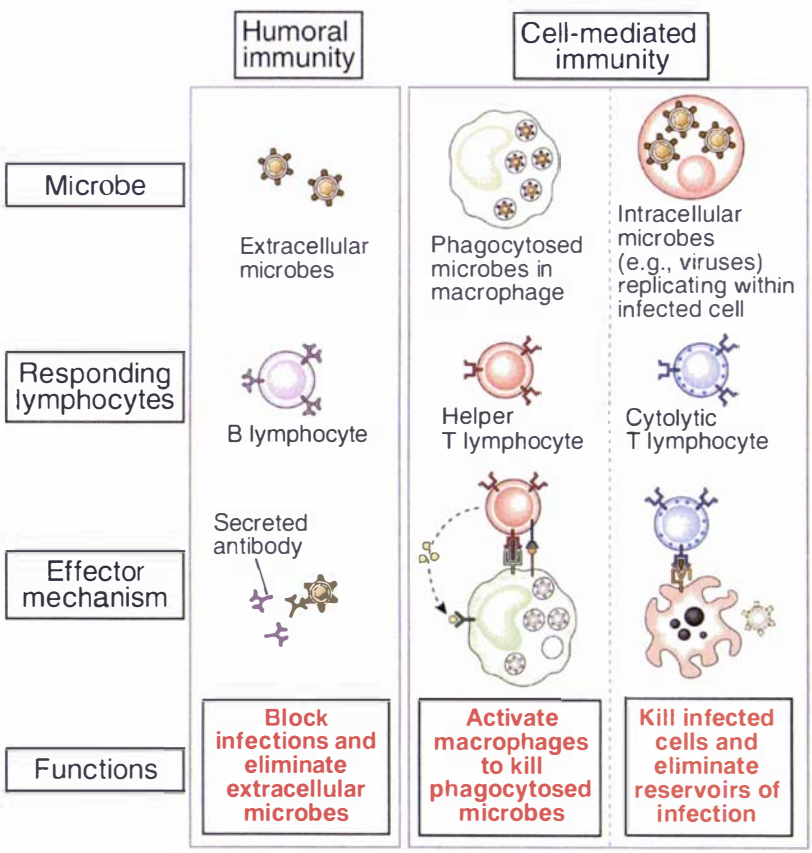

Figure 2.15 - Types and mechanisms of adaptive immunity. This image was taken from Abbas and Lichtman, 2004.

The humoral immune system is mediated by antibodies, which are proteins produced by B lymphocytes. B lymphocytes are located in the lymphoid organs and bone marrow. They secrete antibodies into the circulation and muscular fluids. These antibodies find, neutralise and eliminate microbes and microbial toxins. Antibodies are able to recognise different types of microbial molecules, including proteins, carbohydrates and lipids. Because antibodies destroy extracellular microbes, they are able to stop cell infections from occurring.

When microbes do infect cells the cell-mediated immune system is required. This system is mediated by two types of T lymphocytes. The first type of T lymphocytes, the helper T lymphocytes, activate phagocytes to destroy ingested microbes. The second type of T lymphocytes, the cytolytic T lymphocytes, kill any type of host cell that contain infectious microbes in their cytoplasm. T lymphocytes recognise peptide fragments of protein antigens displayed on other cells; however, unlike antibodies they are not able to recognise other types of molecules.

The first major difference in the adaptive, compared to the innate, immune system is the ability for it to modify. The adaptive immune system has the ability to respond to previously unseen molecules. This can be seen in Figure 2.16, where the B lymphocytes produce antibodies to a new antigen challenge.

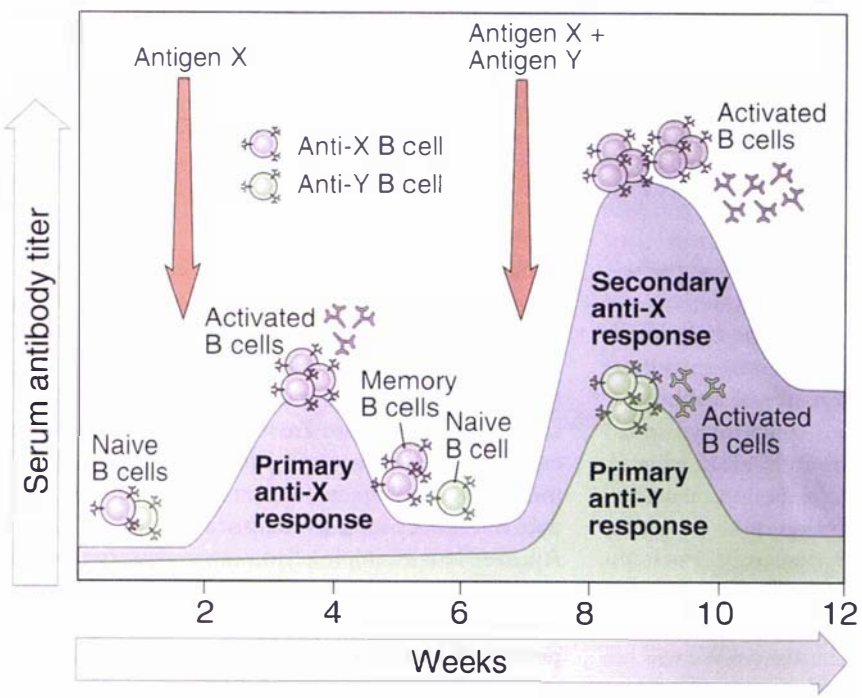

Figure 2.16 - Specificity and memory in adaptive immunity illustrated by primary and secondary immune response. This image was taken from Abbas and Lichtman, 2004.

Another major difference between the adaptive immune system and the innate immune system is that the adaptive immune system is specific. This means that the adaptive immune system is able to distinguish between at least a billion different antigens or portions of antigens, and act accordingly; whereas the innate immune system responds the same for all antigens. This is illustrated in Figure 2.16, which shows that the two antigens induce the production of different antibodies.

Another property of the adaptive immune system that makes it different to the innate immune system is its memory. The adaptive immune system mounts larger and more effective responses to repeated exposures to the same antigen. The first time the system is exposed to a new antigen it mounts a primary immune response by naïve lymphocytes. Subsequent encounters with the same antigen lead to a secondary immune response by memory lymphocytes, which is more rapid, larger and better able to eliminate the antigen than the primary response.

The final important property of the adaptive immune system is its ability to discriminate between "self" and "non-self" molecules. It is able to recognise and respond to molecules that are foreign and avoid making a response to those molecules that are part of the host.

2.3.3 Role of antimicrobial peptides in animal immune systems

Although antimicrobial peptides were originally identified due to their antimicrobial properties, subsequent research has shown that they have a number of other roles within the immune system of animals (Hancock, 2001a; Kamysz *et al*, 2003; Bals and Wilson, 2003). Antimicrobial peptides can be found in concentrations high enough to inhibit microbes at some sites within the host, eg mammalian neutrophil granules and insect lymph fluid; however, in other body sites, eg airway surface fluids, the concentration of antimicrobial peptides is too low to be directly microbicidal. This implies that these peptides carry out other tasks within the host than just direct killing of microbes (Hancock, 2001a). The six proposed roles of antimicrobial peptides within the animal immune system are illustrated in Figure 2.17.

The first role of antimicrobial peptides within the animal immune system is to inhibit microbes. Evidence for the antimicrobial function of these peptides *in situ* was illustrated by a study that used "gene knockout" mice that lacked matrilysin which meant they were able to synthesise and secrete only inactive α-defensins (Wilson and Ouellette, 1999). These mice

had a significantly decreased ability to clear infections compared to wild-type mice. It has also been shown that the human cathelicidin LL-37 is induced in skin cells during damage. During these times LL-37 is produced in concentrations high enough to inhibit microbes (Frohm *et al*, 1997).

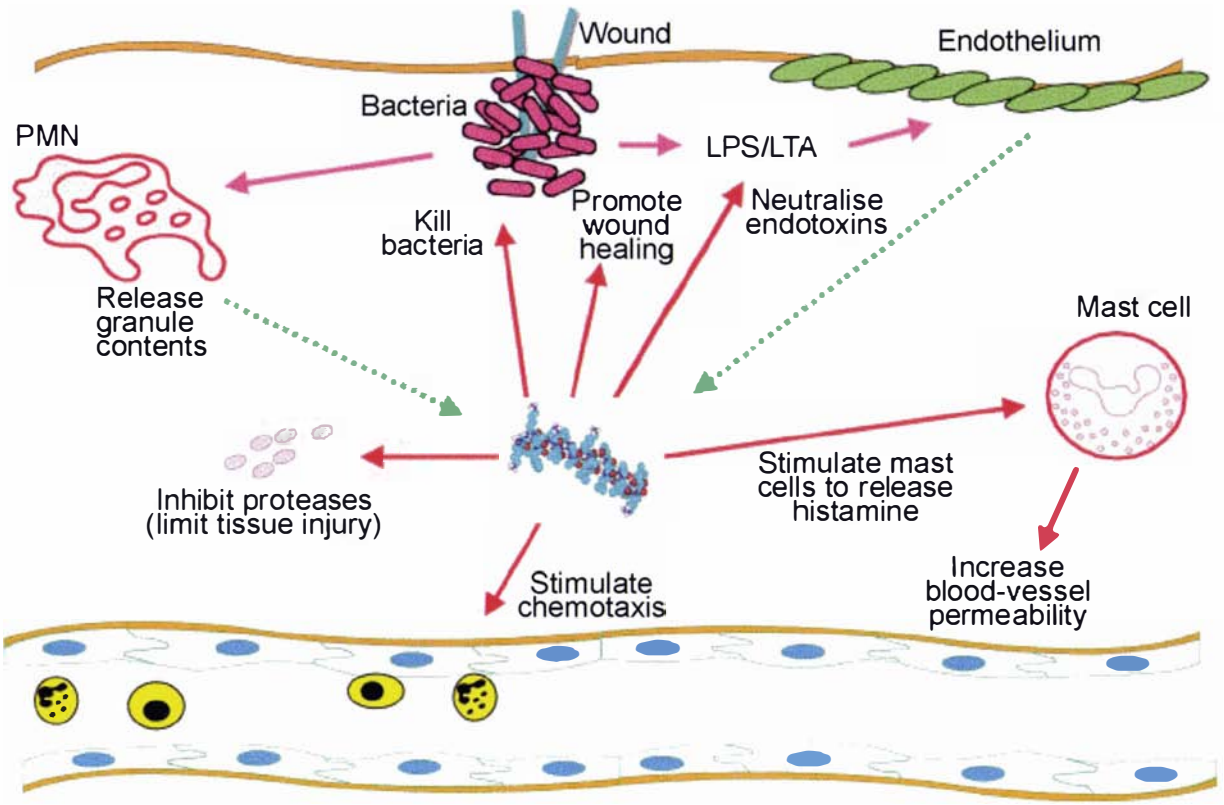

Figure 2.17 - Proposed roles of antimicrobial peptides within the innate immune system. LPS is lipopolysaccharide, LTA is lipoteichoic acid and PMN is polymorphonuclear leukocytes. This image was adapted from that of Hancock, 2001a.

The second role of animal antimicrobial peptides is to reduce host response to bacterial signalling molecules such as endotoxin lipopolysaccharide from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria. These molecules interact with Toll-like receptors on the surface of host cells to trigger signalling cascades and cause upregulation of proinflammatory cytokines. By inhibiting the binding of the bacterial molecules to the host cells, antimicrobial peptides protect the host against endotoxemia (Hancock, 2001a). This was shown in a study using mice with antibiotic-induced endotoxin shock. The mice were protected from death when injected with the a fragment of the murine cathelicidin CAP18 (Kirikae *et al*, 1998).

The third role of animal antimicrobial peptides is to chemoattract cells involved in the innate and adaptive immune systems. It has been shown that some antimicrobial peptides can attract

monocytes (Verbanac *et al*, 1993), neutrophils (Huang *et al*, 1997) and T lymphocytes (Agerberth *et al*, 2000; Yang *et al*, 2000). The chemoattractant properties of antimicrobial peptides are inhibited by pertussis toxin. This means that the antimicrobial peptides must interact with the G protein-coupled receptors (Oppenhei *et al*, 2003). The release of antimicrobial peptides from neutrophils so that they can have their chemoattractant effect is stimulated by interleukin-8 (Chertov *et al*, 1996).

The fourth role of animal antimicrobial peptides is to promote the release of histamine from mast cells. Both defensins and cathelicidins are able to stimulate mast cells to mobilize intracellular Ca^{2+} and release histamine (Yamashita and Saito, 1989; Niyonsaba *et al*, 2001). This histamine modulates inflammatory reactions and increases the permeability of blood vessels.

The fifth role of animal antimicrobial peptides is to inhibit tissue proteases. It has been shown that the bovine cathelicidin peptide precursor proBac5 can inhibit the activity of cathepsin L (Verbanac *et al*, 1993). This proteinase is thought to contribute to tissue injury during inflammation. Similarly, the porcine cathelicidin PR-39 is able to attenuate tissue damage by inhibiting NADPH oxidase, which in turn suppresses superoxide release (Shi *et al*, 1996b; Ikeda *et al*, 2001), and by blocking the stimulation of NF-κB-dependent gene expression (Gao *et al*, 2000).

The final role of animal antimicrobial peptides is to stimulate wound healing. Defensins are able to induce the proliferation of cultured lung cells, which indicates that they may be involved in the repair of airways (Aarbiou *et al*, 2002). The human cathelidicin LL-37 stimulates the proliferation and formation of vessel-like structures in cultured endothelial cells, and the murine cathelicidin CRAMP is important in inducing vascularisation for skin wounds in mice (Koczulla *et al*, 2003).

2.4 POTENTIAL APPLICATIONS OF ANTIMICROBIAL PEPTIDES

Because antimicrobial peptides can influence cellular functions, they are classed as bioactive compounds (Kris-Etherton *et al*, 2004). Bioactive compounds have been isolated from a variety of sources and can have numerous different structures. Examples of bioactive compounds include polyphenols, phytoestrogens, phytosterols, phytates, lectins, oligosaccharides and polyunsaturated fatty acids (Orzechowski *et al*, 2002).

Bioactive peptides are one type of bioactive compound. As well as antimicrobial activity, bioactive peptides can exhibit numerous other properties (Meisel, 1998). These activities include opiate (Teschemacher *et al*, 1997; Bodnar and Hadjimarkou, 2003; Gentilucci, 2004), antithrombotic or anti-hypertensive (Takano, 1998; Yamamoto *et al*, 2003), immunomodulation (Kayer and Meisel, 1996; Meisel and FitzGerald, 2003) and mineral utilisation (FitzGerald, 1998; Meisel and FitzGerald, 2003). The majority of these bioactive peptides are derived from milk proteins by enzymatic hydrolysis, fermentation of milk with proteolytic starter cultures, or action of enzymes derived from proteolytic microorganisms (Korhonen and Philaanto, 2003). The antimicrobial peptides of interest in this research are also cleaved from larger proteins; however, this process occurs naturally, as discussed in Section 2.2.

There has been a large amount of interest in antimicrobial peptides because of their potential to be used in commercial applications, especially for health purposes. The applications of antimicrobial peptides that have been investigated are reviewed and the potential applications of antimicrobial peptides isolated from ovine blood are discussed.

2.4.1 Applications for antimicrobial peptides

Due to the broad-spectrum antimicrobial activity of antimicrobial peptides their applications, particularly as human therapeutics, have been explored. Their use against health problems such as cystic fibrosis (Saiman *et al*, 2001), infected burns (Steinstraesser *et al*, 2002), sexually-transmitted diseases (Borenstein *et al*, 1991; Yasin *et al*, 1996; Qu *et al*, 1996) oral mucositis (Chen *et al*, 2000), endotoxin-induced septic shock (Giacometti *et al*, 2004), viral infections (Daher *et al*, 1986; Yasin *et al*, 2000; Bastian and Schafer, 2001), HIV (Robinson *et al*, 1998), ocular infections (Schuster and Jacob, 1992), tumours (Winder *et al*, 1998; Johnstone *et al*, 2000) and candidiasis (Lupetti *et al*, 2002) have been investigated. Other health-related applications that have been studied include contraception (Sawicki and Mystkowska, 1999), radio-labelled antimicrobial peptides for imaging infections (Nibbering *et al*, 1998) and antimicrobial peptides as membrane translocation mediators for impermeable effector molecules (Fischer *et al*, 2001).

The main motivation for the research into antimicrobial peptides as human therapeutics is the increase in antibiotic resistance strains of bacteria, particularly in hospitals. Due to this problem, new antimicrobial agents, such as antimicrobial peptides, are required to combat

these infections. Because antimicrobial peptides use different mechanisms of action to conventional antibiotics, they are active against antibiotic-resistant organisms. There is, however, concern that antimicrobial peptide-resistant strains may emerge in the future. Some bacterial strains are naturally resistant to antimicrobial peptides because of modifications in their membrane components such as Gram-negative LPS (Groisman *et al*, 1992; Guo *et al*, 1998; Ernst *et al*, 1999; Lysenko *et al*, 2000), Gram-positive teichoic acids (Peschel *et al*, 1999) and membrane lipids (Peschel *et al*, 2001), or because of production of proteases (Groisman *et al*, 1992) (Guina *et al*, 2000; Sieprawska-Lupa *et al*, 2004). However, it is difficult to induce resistance to antimicrobial peptides in the laboratory using techniques such as multiple passages at half the minimum inhibitory concentration, that readily induce resistance to conventional antibiotics (Hancock and Rozek, 2002).

Most research groups have endeavoured to create pharmaceutical products with potent antimicrobial activity and limited human toxicity. To achieve this, these research groups have used naturally occurring antimicrobial peptides as templates for designing new peptides with the desired properties (Andreu *et al*, 1992; Blondelle and Houghten, 1992; Iwata *et al*, 1994; Zhong *et al*, 1995; Mayo *et al*, 2000; Shin *et al*, 2001c), instead of using the natural peptides. This has led to numerous studies to compare the structural features of antimicrobial peptides such as charge, hydrophobicity, amphiphilicity, and α-helicity, to their function (Dathe *et al*, 1996; Bechinger, 1997; Hwang and Vogel, 1998; Juvvadi *et al*, 1999; Powers and Hancock, 2003). From this, it has been determined that amphiphilicity is the most important factor when determining the antimicrobial activity of a peptides (Lee *et al*, 1986; Perez-Paya *et al*, 1995; Pathak *et al*, 1995; Kondejewski *et al*, 1999). Increasing the charge results in the peptides being more specific for bacteria (Dathe *et al*, 1996); whereas, increasing the hydrophobicity increases the cytotoxicity of the peptides (Weipresht *et al*, 1997; Dathe *et al*, 1997).

A number of antimicrobial peptides whose designs were based on natural animal antimicrobial peptides have reached clinical trials. A promising peptide, MSI-78 produced by Magainin Pharmaceuticals Inc, which is based on amphibian magainins, recently underwent clinical trials for the treatment of infected diabetic foot ulcers; however, it failed Phase III and did not get US FDA approval (Koczulla and Bals, 2003). Similarly, the protegrin-derived peptide IB-367 produced by Intrabiotics Pharmaceuticals Inc, failed phase II clinical trials for oral mucositis (Bellm *et al*, 2000; Koczulla and Bals, 2003). This peptide is currently

undergoing clinical trials for the treatment of ventilator-associated pneumonia and cystic fibrosis (www.intrabiotics.com). Another company Migenix (formally known as Micrologix) currently has MX-226 in phase III clinical trials for the treatment of catheter-related infections and MX-1121 that completed phase II clinical trials for the treatment of acne (www.migenix.com). A new company Inimex Pharmaceuticals Inc aims to develop antimicrobial peptides that enhance innate immune responses (Finlay and Hancock, 2004)(www.inimexpharma.com).

2.4.2 Possible applications for ovine blood antimicrobial peptides

The research presented in this thesis aimed to investigate the properties of ovine blood antimicrobial peptides with the objective of establishing their suitability in value-added products. This meant that unlike the research previously carried out, which uses mostly synthetic peptides as potential products, this research was concerned with uses for the naturally occurring peptides isolated from ovine blood.

Three possible uses for antimicrobial peptides isolated from ovine blood were identified. Firstly, the peptides could be used as treatments for diseases in sheep and other animals. Secondly, they could be used as natural biopreservatives for chilled meat products to increase the shelf life and decrease waste. Thirdly, they could be isolated and sold as value-added products for human therapeutic uses.

The first possible application for antimicrobial peptides isolated from ovine blood is in the treatment of animal diseases. Some research has been carried out on the effectiveness of ovine peptides as ovine therapeutics. To date, the research has mostly focused on the interactions of antimicrobial peptides with *Mannheimia (Pasteurella) haemolytica.* This bacterium is an ovine respiratory pathogen, which if uncontrolled can proliferate throughout the upper respiratory tract and lungs and lead to acute pneumonia. It is thought that ovine β-defensins play a natural role in restricting the spread of this pathogen (Brogden *et al*, 1998).

The effectiveness of the ovine α-helical cathelicidins, SMAP29 and SMAP34, against *Mannheimia haemolytica* and other ovine pathogens, including *Pseudomonas aeruginosa,* have been investigated (Brogden *et al*, 2001). Both peptides showed activity against a wide range of bacteria, with SMAP29 being the more active of the two. This same study demonstrated that SMAP29 reduced the concentration of bacteria in ovine bronchoalveolar lavage fluid and pulmonary tissues. This suggests that it may be an effective treatment;

however, further studies are needed to determine the dosage amount and frequency of administration required.

The second possible application for antimicrobial peptides isolated from ovine blood is as biopreservatives for chilled meat products. For this to be viable, a number of factors have to be considered, including safety, efficacy, consumer-acceptance and cost-effectiveness.

The first issue to consider is the safety of the product. Since peptides are proteinaceous, it is likely that they would be degraded to amino acids in the digestive system and would not be toxic to humans; however, tests are required to verify this. Similar peptides derived from microbial sources, such as nisin, are already routinely used as food preservatives.

Secondly, it needs to be determined if the peptides work affectively as a meat preservative. There are no reports concerning antimicrobial peptides as meat preservatives so research into their efficacy needs to be carried out. It is hoped that these peptides will decrease the microbial load on chilled meat prior to packaging and, therefore, increase the shelf life of the product. Some of the ovine cathelicidins, such as SMAP29, are effective at physiological salt concentrations (Travis *et al*, 2000); however more tests into the factors that affect the activity of the peptides are required.

The main competitor for antimicrobial peptides in the biopreservative industry would be bacteriocins (Kim, 1993; Stiles, 1994; Holzapfel *et al*, 1995). Bacteriocins are peptide antibiotics produced by bacteria to inhibit competing bacteria. As discussed in Section 2.2.3, these peptides often have only a narrow spectrum of antimicrobial activity and do not usually inhibit Gram-negative bacteria. However, some Gram-negative bacteria can cause food poisoning, for example *E. coli* O157:H7. One bacteriocin that does have broad-spectrum activity and is currently being used as a food preservative is nisin, which is produced by some strains of *Lactococcus lactis* (Shin *et al*, 2000b).

Some testing has been carried out with nisin as a meat preservative but the results are conflicting. One study using nisin as a preservative for raw buffalo meat mince showed that it is effective at inhibiting the growth of *Listeria monocytogenes,* and that the activity was increased if nisin was used in conjunction with lactic acid (Barbuddhe *et al*, 1999). However, a similar test on vacuum-packaged fresh beef showed nisin was not an effective preservative and nisin in combination with lactic acid and polylactic acid did not significantly increase the effect of the acids (Ariyapitipun *et al*, 1999).

Other bacteriocins have also been investigated. Lacticin 3147, derived from *Lactococcus lactis* DPC 3147, was active as a biopreservative in fresh pork sausages and its activity was increased with the addition of sodium citrate or sodium lactate (Scannell *et al*, 2000). A cell preparation from *Pediococcus acidilactici* H (pediocin AcH) inhibited *Listeria monocytogenes* on raw chicken meat and was still active after cooking (Goff *et al*, 1996). Enterocins A and B inhibited the growth of *Listeria innocua* in a variety of meats and meat products (Aymerich *et al*, 2000).

Another possibility is to use the peptides in antimicrobial food packaging (Han, 2000; Quintavalla and Vivini, 2002). When nisin was incorporated in plastic packaging it was able to inhibit the surface growth of bacteria on meat (Siragusa *et al*, 1999), which shows that animal antimicrobial peptides may also be active when incorporated into food packaging.

The third important factor in determining the success of antimicrobial peptides as biopreservatives is consumer acceptance. If the peptides were to be used as general preservatives there may be problems because they are from animal sources so they would not be suitable for vegetarians. There may also be concerns about the fact that the peptides are derived from blood, but this may be overcome by using blood from countries with animals that are free of troublesome diseases, such as New Zealand. Using the peptides as biopreservatives for chilled meat of the animal they were derived from may help minimise these concerns. In this case, only people who are already comfortable with consuming meat would ingest the peptides, and since the peptides are a natural part of the animal there should be little objection.

The final important factor to consider is the economic viability of isolating the peptides. The economic gain from the use of the peptides to extend the shelf life of chilled meat products needs to be greater than the cost of isolating the peptides. Since the peptides would be used as a biopreservative, high purity is not important. A crude extract, containing a mixture of the antimicrobial peptides as well as other blood proteins, could be used. This would eliminate the need for expensive downstream processing steps. Another way to make the process more viable would be to separate other valuable products simultaneously from the blood, for example blood serum (used in laboratories for growth of cell cultures) and blood proteins (serum albumins, fibronectin, transferrin, antibodies, trypsin etc).

The third possible application for antimicrobial peptides isolated from ovine blood is as therapeutics for humans. Most of the research into antimicrobial peptides has been driven by the need to find alternative therapeutics to overcome antibiotic-resistant organisms, for example methicillin resistant *Staphylococcus aureus* (MRSA), which cause troublesome infections in hospitals. The use of antimicrobial peptides as pharmaceuticals has been widely researched; however, the existing work has usually focussed on natural antimicrobial peptides as models to design therapeutic peptides (Hancock, 2000).

Ovine antimicrobial peptides could be used as either pharmaceuticals or nutraceuticals. Pharmaceuticals are higher value products; however, they require expensive clinical trials and a very high purity. This means the peptides would need to be produced artificially. Nutraceuticals are products that are available in health food stores. These products are lower value than pharmaceuticals; however, they do not require clinical trials to establish proof of efficacy or such a high purity. This would reduce the production cost dramatically and utilise the naturally occurring peptides isolated from livestock blood.

For a nutraceutical product, an extract containing a mixture of ovine antimicrobial peptides in the form of a topical cream could be used due to its wide applications and ease of use. This cream could be applied to protect small cuts and grazes from infection, or possibly as a treatment for fungal infections such as athlete's foot. However, research is required to test the efficacy of this concept.

For a pharmaceutical product, the ovine candidate with the greatest potential is SMAP29, due to its potent broad-spectrum activity. SMAP29 has unchanged minimum inhibitory concentrations against *E. coli, S. aureus* and *P. aeruginosa* after 20 passages with sublethal concentrations (Hashimoto *et al*, 1993). Therefore, resistance to this peptide does not easily develop. Tests have shown that SMAP29 is effective *in vitro* at killing strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients (Saiman *et al*, 2001; Saiman *et al*, 2001). This peptide has also proven to be active against a wide variety of oral pathogens, so it can potentially be used to treat oral infections (Fuse *et al*, 1993). Although the results are encouraging, the testing of SMAP29 *in vivo* has not been reported to date.

Another possibility is to use the most potent livestock peptides as lead compounds in the design of new pharmaceuticals. The sheep cathelicidin SMAP29 has been used as a model in the design of a new peptide called ovispirin (Kalfa *et al*, 2001). This peptide and many of its

derivatives showed broad-spectrum activity similar to that of SMAP29 (Robinson *et al*, 1998; Saiman *et al*, 2001; Kalfa *et al*, 2001). OV-1, an 18-residue peptide based on ovispirin shortened from both ends, was the most active of these new peptides. However, this peptide was also highly toxic to human cells, leading to the design of two new peptides termed novispirins (Sawai *et al*, 2002). These peptides differed from OV-1 by only one residue, but they both had greatly reduced cytotoxicity while retaining their antimicrobial activity, which makes them excellent candidates for therapeutics. Novispirin G10 was tested on rat burns that were infected with *P. aeruginosa*. The results showed a significant reduction in the number of bacteria present (Steinstraesser *et al*, 2002). Further studies are required to determine whether novispirins can be effective at treating burns, or other wound infections, in humans.

For the commercial production of SMAP29, and/or its derivatives, transgenic microorganisms, plants, or animals could be used instead of costly chemical synthesis. Important factors to consider in transgenic systems are correct processing and cleavage, prevention of proteolytic degradation, and sensitivity of the host. Due to these factors recombinant production of antimicrobial peptides in microorganisms has had limited success (Rautenbach and Hastings, 1999).

The second option is to produce the antimicrobial peptide in transgenic plants. One study has described the successful production of SMAP29 in tobacco plants (Morassutti *et al*, 2002). The peptide was purified by passing the crude leaf extract through an affinity column.

The final option is the production of the antimicrobial peptide in transgenic animals. A bovine β-defensin has been successfully expressed in the milk of transgenic mice, showing that mammary glands can be used as bioreactors to produce antimicrobial peptides (Yarus *et al*, 1996). The peptide was purified from the milk using acid precipitation, ion-exchange chromatography and reverse-phase high performance liquid chromatography (RP-HPLC), which is a relatively simple process. For the large-scale production of SMAP29, transgenic sheep expressing the peptide in their milk could be used. This would be beneficial for the lamb industry from which the peptides were first derived.

2.5 PURIFICATION AND CHARACTERISATION OF ANIMAL ANTIMICROBIAL PEPTIDES

The first objective of the research in this thesis was to purify and characterise antimicrobial peptides from ovine blood. As discussed in Section 2.2.1, antimicrobial peptides have been

isolated from a variety of locations within animals including the blood, respiratory tract, gastrointestinal tract, urogenital tract and skin. Because the research presented in this thesis focussed on antimicrobial peptides from ovine blood, the techniques used to isolate antimicrobial peptides from blood, as opposed to other sites, was the focus of the investigation. Other techniques, such as those used to characterise and test the activity of the antimicrobial peptides, were common between all the antimicrobial peptides regardless of their location within the host.

The current knowledge regarding the antimicrobial peptides known to be located within the blood of livestock animals is reviewed. The limitation of host animals to livestock was necessary because such a large number of antimicrobial peptides are now known. The gene-encoded antimicrobial sequences database hosted by the Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Italy, online at http://www.bbcm.univ.trieste.it/~tossi/pag1.htm, which is updated regularly, lists more than 800 antimicrobial peptides. Livestock animals, including cattle, pigs and goats were chosen as the focus group because they are evolutionarily related to sheep and, therefore, more likely to contain similar antimicrobial defence systems.

Finally, the previous work carried out to purify and characterise antimicrobial peptides from ovine blood is reviewed. From this, areas where knowledge was lacking were identified, so that the specific objectives of the current research project could be formulated.

2.5.1 Techniques to purify and characterise antimicrobial peptides

In order to purify and characterise antimicrobial peptides from blood a number of steps are required. Firstly, the antimicrobial peptides are extracted from the blood neutrophils, and then they are purified from the crude extract. Once the antimicrobial peptides are purified, their structures are determined and their antimicrobial activity is tested. For each of these steps a few different experimental techniques have been reported in the literature.

A few different techniques have been described for the extraction of antimicrobial peptides from blood. The steps involved are the removal of the red blood cells, isolation of the white blood cells, isolation of the granules, and then extraction of the antimicrobial peptides. These steps are present in all the techniques, but the methods used are sometimes different.

In most cases the blood is centrifuged to collect the blood cells fraction, then the red blood cells are lysed (Carlson and Kaneko, 1973). The solutions used for lysing and restoring the isotonicity vary between groups. Most commonly, deionised water is used to lyse the red blood cells (Gennaro *et al*, 1983), but in some instances other solutions such as 0.2% NaCl (Yamashita and Saito, 1989) are used. The isotonicity is restored with numerous different buffers including phosphate buffered 2.7% saline (Carlson and Kaneko, 1973). Alternative methods include the sedimentation of red blood cells from whole blood with 3% dextran sulphate (Tang *et al*, 1999a) and the lysis of red blood cells in whole blood with 0.83% ammonium chloride, which do not require the isotonicity to be restored afterwards (Dioguardi *et al*, 1963; Eggleton *et al*, 1989).

In most cases the white blood cells are isolated by collecting them via centrifugation, but the solutions used differ between groups. Solutions used include washing the white blood cells in phosphate-buffered saline and resuspending the white blood cells in 0.34M sucrose containing 0.5mM $MgCl_2$ (Gennaro *et al*, 1983); and resuspending the white blood cells in 0.05M sodium phosphate buffer containing 2mM phenylmethylsulfonylfluoride, 10μM leupeptin, 10μM pepstatin, 1mM *n*-ethylmaleimide and 2mM EDTA (Eisenhauer *et al*, 1989);

The neutrophil granules have been isolated from the white blood cells by a few different techniques. The white blood cells can be disrupted by either using homogenisation (Gennaro *et al*, 1983) or sonication (Eisenhauer *et al*, 1989). Then the granules can be collected via a discontinuous density gradient (Gennaro *et al*, 1983) or centrifugation (Eisenhauer *et al*, 1989).

The antimicrobial peptides can be extracted from the neutrophil granules by a number of techniques including suspending the granules in 0.2M sodium acetate for 2 hours with continuous stirring (Gennaro *et al*, 1983), suspending the granules in 20% acetic acid for one hour at 4°C with stirring (Eisenhauer *et al*, 1989), and suspending the granules in 10% acetic acid overnight at 4°C with stirring (Selsted *et al*, 1993).

There have also been a few different techniques used for the purification of the antimicrobial peptides from the crude extract. In most cases the crude extract was fractionated by ion-exchange chromatography (CM-cellulose (Gennaro *et al*, 1989)), or gel filtration (Sephadex G-50 (Gennaro *et al*, 1983; Savoini *et al*, 1984); Bio-Gel P10 (Eisenhauer *et al*, 1989; Evans *et al*, 1994), Bio-Gel P-60 (Selsted *et al*, 1992; Shi *et al*, 1994)), before being further purified

by RP-HPLC (Selsted, 1997). Another technique separated the peptides by preparative PAGE then excised and eluted the bands of interest (Yamashita and Saito, 1989).

After the peptides have been purified, the next step is to determine their structures. This has been done to different extents by different research groups. In most cases the mass of the peptides and at least the N-terminal sequence, if not the whole amino acid sequence, are determined (Selsted and Harwig, 1987; Harwig *et al*, 1995). In some cases the secondary structure is also determined using techniques such as circular dichroism (CD) (Johansson *et al*, 1998; Park *et al*, 2002) and nuclear magnetic resonance (NMR) (Zimmermann *et al*, 1995).

In the literature there are three main techniques used to test the antimicrobial activity of antimicrobial peptides. These are the radial diffusion plate assay, the gel overlay and the micro-broth dilution methods. The radial diffusion plate assay method is used both to indicate the present or absence of antimicrobial activity and to quantify the antimicrobial activity. In contrast, the gel overlay method is used to test only for the presence or absence of antimicrobial activity and the micro-broth dilution method was used to determine only the minimum inhibitory concentration (MIC) of a substance.

The first technique used to test the antimicrobial activity of antimicrobial peptides is the radial diffusion plate assay method (Lehrer *et al*, 1991; Steinberg and Lehrer, 1997). In this method the test culture is added to an underlay agar that is poor in nutrients. Wells are made in the agar and the substances to be tested are added to the wells. The test substances are left to diffuse through the agar and then an overlay agar that is rich in nutrients is added to the top. The test culture grows between the two layers of agar but it does not grow in areas where the antimicrobial substances are present. This technique has been use to test whether the crude extract has antimicrobial activity and to test which fractions are active after separation steps (Evans *et al*, 1994; Shi *et al*, 1994; Tang *et al*, 1999a). This technique has also been used to determine the potency of antimicrobial peptides (Harwig *et al*, 1993; Harwig *et al*, 1995; Takemura *et al*, 1996). To do this, different concentrations of the peptides are tested in the radial diffusion assay. The diameter of the clearing sizes are plotted against the log of the peptide concentration and a straight line is fitted. From the equation of the line the x-intercept is calculated. This is the peptide concentration where the clearing size is zero so it is the MIC.

The second technique used to test the antimicrobial activity of the antimicrobial peptides is the gel overlay method (Lehrer *et al*, 1991; Steinberg and Lehrer, 1997). This method is used to test which components separated by gel electrophoresis have antimicrobial activity. A mixture of proteins and peptides, usually the crude neutrophil extract, are subjected to polyacrylamide gel electrophoresis (PAGE). Once the components are separated, the polyacrylamide gel is placed on underlay agar containing the test organism for three hours to allow the peptides to diffuse into the agar. Once the polyacrylamide gel is removed, the overlay agar, rich in nutrients, is poured onto the top. After overnight incubation, zones of inhibition can be seen in areas where the antimicrobial peptides are present. This can then be compared to an identical gel to determine which bands have antimicrobial activity.

The third technique used to test the antimicrobial activity of the antimicrobial peptides is the micro-broth dilution method. This method is used to determine the MIC of antimicrobial peptides. This involves the testing of serial dilutions of the antimicrobial substance against a standard concentration of microbial cells. The lowest concentration of the test substance that is able to inhibit the growth of the test organism overnight is considered the MIC. This method is recommended by the National Committee of Laboratory Safety Standards (NCLSS) (Amsterdam, 1996). Modifications to this assay specifically for cationic peptides were recommended by Professor Robert Hancock (Department of Microbiology and Immunology, University of British Columbia) at the First Gordon Conference of Antimicrobial Peptides in 1996 (Hancock, personal communication). The first modification suggested by Hancock was the use of polypropylene instead of polystyrene 96-well plates to avoid the cationic peptides binding to polystyrene plates, which may affect either activity. The second modification suggested by Hancock was the use of Mueller-Hinton broth (MHB) instead of tryptic-soy broth (TSB) because the high salt concentration in TSB can affect the activity of the peptides. Hancock refers to this technique as the 'gold standard' for antimicrobial peptide testing (Hancock, 2001a).

2.5.2 Livestock blood antimicrobial peptides

A number of defensins and cathelicidins have been identified in blood cells of livestock animals. Some of these have been purified from blood, whereas others have been predicted from cDNA sequences.

Of the three types of defensins discussed in Section 2.2.1, only β-defensins have been discovered in the blood of livestock. Thirteen β-defensins have been identified in the neutrophils of cattle (Selsted *et al*, 1993), two in chickens (Evans *et al*, 1994; Brogden *et al*, 1999), one in turkey (Evans *et al*, 1994), and one in ostrich (Yu *et al*, 2001). These β-defensins are active against Gram-positive and Gram-negative bacteria (Selsted *et al*, 1993), but only at low salt concentrations (Bals *et al*, 1998). The amino acid sequences of these peptides are given in Table 2.1.

As well as the defensins, numerous cathelicidins of different structural types have been identified in the blood of different livestock animals. The first structural class of cathelicidins is rich in one or more amino acids. Twelve cathelicidins rich in one or more amino acids have been found in livestock animals. The amino acid sequences of these peptides are given in Table 2.2.

Of these peptides, nine are rich in both proline and arginine. Three proline/arginine rich peptides are found in cattle, Bac4, Bac5, Bac7 (bactenecins with molecular weights of 4, 5, and 7kDa) (Gennaro *et al*, 1983; Gennaro *et al*, 1989), four in sheep, OaBac5, OaBac6, OaBac7.5, and OaBac11 (*Ovine aries* bactenecins with molecular weights of 5, 6, 7.5 and 11kDa) (Bagella *et al*, 1995; Huttner *et al*, 1998); one in goats, ChBac5 (*Capra hircus* bactenecin with a molecular weight of 5kDa) (Shamova *et al*, 1999); and one in pigs, PR-39 (proline-arginine rich peptide with 39 amino acids) (Storici and Zanetti, 1993). These peptides are active against a wide range of organisms but they are inhibited by high salt concentrations (Skerlavaj *et al*, 1990).

As well as the cathelicidins that are rich in both proline and arginine, there are others that are rich in other amino acids. Prophenin is a porcine peptide rich in only proline (Harwig *et al*, 1995) whereas PMAP-23 is a porcine peptide rich in only arginine (Zanetti *et al*, 1994). Prophenin contains an unusually large amount of proline (53.2%) and is more active against Gram-negative bacteria than Gram-positive bacteria (Harwig *et al*, 1995). PMAP-23 is active against Gram-positive and Gram-negative bacteria as well as cancer cells without being haemolytic (Shin *et al*, 2000a). The last peptide in this group is a bovine peptide, indolicidin, which is rich in tryptophan (Selsted *et al*, 1992). This peptide is active against Gram-positive and Gram-negative bacteria, as well as fungi, protozoa (Aley *et al*, 1994) and the HIV virus (Robinson *et al*, 1998). However, it is also highly cytotoxic making it unsuitable for therapeutic purposes.

Table 2.1 - Amino acid sequences of the β-defensins found in livestock blood.

Peptide	Source	Sequence	Reference
BNDB-1	cattle	DFAS**C**HTNG**G**I**C**LPNR**C**PGHMIQI**G**I**C**FRPRVK**CC**RSW	Selsted *et al*, 1993
BNDB-2	cattle	VRNHVT**C**RINR**GFC**VPIR**C**PGRTRQI**GTC**FGPRIK**CC**RSW	
BNDB-3	cattle	PEGVRNHVT**C**RINR**GFC**VPIR**C**PGRTRQI**GTC**FGPRIK**CC**RSW	
BNDB-4	cattle	PERVRNPQS**C**RWNM**G**V**C**IPFL**C**RVGMRQI**GTC**FGPRVP**CC**RR	
BNDB-5	cattle	PEVVRNPQS**C**RWNM**G**V**C**IPIS**C**PGNMRQI**GTC**FGPRVP**CC**RRW	
BNDB-6	cattle	PEGVRNHVT**C**RIYG**GFC**VPIR**C**PGRTRQI**GTC**FGRPVK**CC**RRW	
BNDB-7	cattle	PEGVRNFVT**C**RINR**GFC**VPIR**C**PGHRRQI**GTC**LGPRIK**CC**R	
BNDB-8	cattle	VRNFVT**C**RINR**GFC**VPIR**C**PGHRRQI**GTC**LGPQIK**CC**R	
BNDB-9	cattle	PEGVRNFVT**C**RINR**GFC**VPIR**C**PGHRRQI**GTC**LAPQIK**CC**R	
BNDB-10	cattle	PEGVRSYLS**C**WGNR**GI**C**C**LLNR**C**PGRMRQI**GTC**LAPRVK**CC**R	
BNDB-11	cattle	GPLS**C**RRNG**G**V**C**IPIR**C**PGPMRQI**GTC**FGRPVK**CC**RSW	
BNDB-12	cattle	GPLS**C**GRNG**G**V**C**IPIR**C**PVPMRQI**GTC**FGRPVK**CC**RSW	
BNDB-13	cattle	SGISGPLS**C**GRNG**G**V**C**IPIR**C**PVPMRQI**GTC**FGRPVK**CC**RSW	
CHP-1	chicken	GRKSD**C**FRKS**GFC**AFLK**C**PSLTLIS**GK**C SRFYL**CC**KRIR	Evans *et al*, 1994
CHP-2	chicken	GRKSD**C**FRKN**GFC**AFLK**C**PYLTLIS**GL**C SXFYL**C**	Evans *et al*, 1994
THP-1	turkey	GKREK**C**LRRN**GFC**AFLK**C**PTLSVIS**GTC** SRFQV**CC**	Evans *et al*, 1994
ostricacin-1	ostrich	NNNLF**C** RK**GTC**HFGG**C**PAHLVKV**GSC** FGFRA**CC**KWPWDV	Yu *et al*, 2001

The bold residues are conserved between peptides.

Table 2.2 - Amino acid sequences of cathelicidins rich in one or more amino acids found in livestock blood.

Peptide	Source	Sequence	Reference
Bac4	cattle	RRLHPQHQRFPRERPWPKPLSLPLPRPGPRPWPKPL	Scocchi *et al*, 1998
Bac5	cattle	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP	Gennaro *et al*, 1989
Bac7	cattle	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRPLPFPRPGPRPIPRP	Gennaro *et al*, 1989
OaBac5	sheep	RFRPPIRRPPIRPPFRPPFRPPVRPPIRPPFRPPFRPPIGPFP	Bagella *et al*, 1995
OaBac6	sheep	RRLRPRHQHFPSERPWPKPLPLPLPRPGPRPWPKPLPLPLPRPGLRPWKPL	Huttner *et al*, 1998
OaBac7.5	Sheep	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQPRRIPRPILLPWRPPRPIPRPQPQPIPRWL	Huttner *et al*, 1998
OaBac11	sheep	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRPLPLPRPRPKPIPRPLPLPRPRPRRIPRPLPLPRPRPRPIPRPLPLPQPQPSPIPRPL	Huttner *et al*, 1998
ChBac5	goat	RFRPPIRRPPIRPPFNPPFRPPVRPPFRPPFRPPFRPPIGPFP	Shamova *et al*, 1999
PR39	pigs	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	Storici and Zanetti, 1993
Prophenin	pigs	AFPPPNFPGPRFPPPNVPGPRFPPPNFPGPRFPPPNFPGPRFPPPNFPGPPFPPPIFPGPWFPPPPPFRPPPFGPPRFPGRR	Harwig *et al*, 1995
PMAP-23	pigs	RIIDLLWRVRRPQKPKFVTVWVR	Zanetti *et al*, 1994
indolicidin	cattle	ILPWKWPWWPWRR	Selsted *et al*, 1992

The second structural class of cathelicidins are α-helical. Seven α-helical cathelicidins have been discovered in livestock. The amino acid sequences of these peptides are given in Table 2.3. Three of these were found in cattle, BMAP27, BMAP28 and BMAP34 (bovine myeloid antimicrobial peptides with 27, 28 and 34 residues) (Scocchi *et al*, 1997; De Lucca and Walsh, 1999); two in pigs, PMAP36 and PMAP37 (porcine myeloid antimicrobial peptides with 36 and 37 residues) (Storici *et al*, 1994); and two in sheep, SMAP29/OaMAP29 and SMAP34/OaMAP34 (sheep/*ovine aries* myeloid antimicrobial peptides with 29 and 34 residues) (Mahoney *et al*, 1995; Bagella *et al*, 1995; Huttner *et al*, 1998). These α-helical cathelicidin peptides are active against a broad spectrum of Gram-positive and Gram-negative bacteria, and some are also fungicidal (BMAP27, BMAP28 and SMAP29). The α-helical cathelicidins are haemolytic, but at concentrations much higher than those that are needed for antimicrobial activity.

The final structural class of cathelicidins contain disulphide bonds. Seven cathelicidins containing disulphide bonds have been found in the blood of livestock. The amino acid sequences of these peptides are given in Table 2.4. One of these is from cattle, bactenecin (Romeo *et al*, 1988); one is from sheep, OaDode (*Ovine aries* dodecapeptide) (Huttner *et al*, 1998); and five are from pigs, PG-1, PG-2, PG-3, PG-4 and PG-5 (protegrins 1-5) (Kokryakov *et al*, 1993). Bactenecin and OaDode both contain one disulphide bond. Bactenecin has been shown to be active against Gram-negative bacteria (Romeo *et al*, 1988), but it is more active against Gram-positive bacteria (Wu and Hancock, 1999a). The protegrins contain four cysteine residues that form two disulphide bonds. These peptides are active against Gram-negative and Gram-positive bacteria, fungi and enveloped viruses (Kokryakov *et al*, 1993; Steinberg *et al*, 1997). This activity is retained at physiological salt concentrations (Bellm *et al*, 2000).

Table 2.3 - Amino acid sequences of α-helical cathelicidins found in livestock blood.

Peptide	Source	Sequences	References
BMAP27	cattle	GRFKRFRKKFKKLFKKLSPVIPLLHLG	Skerlavaj *et al*, 1996
BMAP28	cattle	GGLRSLGRKILRAWKKYGPIIVPIIRIG	Skerlavaj *et al*, 1996
BMAP34	cattle	GLFRRLRDSIRRGQQKILEKARRIGERIKDIFRG	Scocchi *et al*, 1997
PMAP36	pigs	GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG	Storici *et al*, 1994
PMAP37	pigs	GLLSRLRDFLSDRGRRLGEKIERIGQKIKDLSEFFQS	Storici *et al*, 1994
SMAP29	sheep	RGLRRLGRKIAHGVKKYGPTVLRIIRIAG	Bagella *et al*, 1995
SMAP34	sheep	GLFGRLRDSLQRGGQKILEKAREIWCKIKDIFRG	Mahoney *et al*, 1995

Table 2.4 - Amino acid sequences of cathelicidins containing disulphide bonds found in livestock blood.

Peptide	Source	Sequences	References
bactenecin	cattle	RLCRIVVIRVCR	Romeo *et al*, 1988
OaDode	sheep	RICRIIFLRVCR	Huttner *et al*, 1998
PG-1	pigs	RGGRLCYCRRRFCVCVGR	Kokryakov *et al*, 1993
PG-2	pigs	RGGRLCYCRRRFCICV	Kokryakov *et al*, 1993
PG-3	pigs	RGGGLCYCRRRFCVCVGR	Kokryakov *et al*, 1993
PG-4	pigs	RGGRLCYCRGWICFCVGR	Kokryakov *et al*, 1993
PG-5	pigs	RGGRLCYCRPRFCVCVGR	Kokryakov *et al*, 1993

2.5.3 Ovine antimicrobial peptides

As discussed, seven antimicrobial peptides have been predicted from ovine cDNA to be present in ovine blood (Mahoney *et al*, 1995; Bagella *et al*, 1995; Huttner *et al*, 1998). These include four proline/arginine-rich peptides, two α-helical peptides and one peptide containing a disulphide bond. However, of these only one peptide has been isolated from ovine blood.

Four proline/arginine-rich cathelicidins were predicted from ovine cDNA. Two variants of one of these predicted peptides, OaBac5, have been isolated from ovine blood (Shamova *et al*, 1999). The purified peptides were amidated and differed from the predicted OaBac5 by at least one amino acid in the case of OaBac5α and at least 3 amino acids for OaBac5β. OaBac5α had broad-spectrum activity. Its MIC values were between 0.5 and 2 μg/mL against a variety of Gram-negative and Gram-positive bacteria. The activity of OaBac5β was not determined in this study as not enough material was available.

Two ovine α-helical cathelicidins, SMAP29 and SMAP34 were predicted from ovine cDNA. To date neither of these peptides has been isolated from ovine blood, but both have been synthesised and studied. The SMAP29 cathelicidin gene was identified by two research groups simultaneously (Bagella *et al*, 1995); however, Mahoney and coworkers were the first to synthesise and test the activity of this peptide (Mahoney *et al*, 1995). It is thought that the natural form of SMAP29 would be an amidated, 28 residue peptide, so it is usually synthesised in this form (Mahoney *et al*, 1995; Bagella *et al*, 1995; Skerlavaj *et al*, 1999).

Numerous studies have shown that SMAP29 has very potent, broad spectrum activity (Skerlavaj *et al*, 1999; Travis *et al*, 2000; Brogden *et al*, 2001; Guthmiller *et al*, 2001). SMAP29 has MIC values in the ranges of 0.5-8 μg/mL for Gram-negative bacteria, 0.5-3 μg/mL for Gram-positive bacteria and 3-12 μg/mL for yeast. Interestingly, SMAP29 is haemolytic against human, but not sheep erythrocytes (Skerlavaj *et al*, 1999). However, the concentrations required to lyse human erythrocytes are ten-fold higher than those needed for antibacterial activity.

Other studies have looked at the relationship between the structure and the function of SMAP29. It was shown that SMAP29 has a random structure in aqueous solutions but forms an α-helix in solutions containing sodium dodecyl sulfate (SDS) micelles (Shin *et al*, 2001b). This same study showed that the N-terminal α-helical region was responsible for the

antimicrobial activity, whereas the C-terminal hydrophobic region was responsible for the haemolytic activity. The proline residue at position 19 was crucial for potent antimicrobial activity. Another study, focusing on the LPS binding ability of SMAP29, showed that it had two LPS binding sites, one in the N-terminal region and one in the C-terminal region and that LPS binds cooperatively (Tack *et al*, 2001). This study also determined that residues 8 to 12 are in a helical structure, residues 18 and 19 form a hinge and residues 20 to 28 make up an ordered hydrophobic segment.

The other ovine α-helical peptide, SMAP34, has not been investigated in the same depth as SMAP29. Like SMAP29, SMAP34 also has broad-spectrum activity, although not as potent. It is also not significantly affected by high salt concentrations (Travis *et al*, 2000; Brogden *et al*, 2001). SMAP34 contains a single cysteine residue in the C-terminal region, which, in the case of guinea pig CAP11, promotes homodimerisation by the formation of an intermolecular disulphide bond (Hashimoto *et al*, 1993).

The study of ovine cDNA uncovered two identical genes that encoded the short peptide, OaDode (*Ovis aries* dodecapeptide) (Huttner *et al*, 1998). This peptide is homologous to the bovine dodecapeptide bactenecin, with only 4 residues differing (Bagella *et al*, 1995). Bovine bactenecin is a small (12 residues), amphipathic loop structure held in place by a disulphide bond. Since OaDode differs by the substitution of hydrophobic residues for different residues that are also hydrophobic, the structure of OaDode is thought to be similar to that of the bovine dodecapeptide. OaDode has not been isolated or synthesised so its activity has not been examined. However, bovine bactenecin is active against *E. coli* and *S. aureus* (Romeo *et al*, 1988).

β-defensins have also been identified in sheep; however they are not stored in the blood neutrophils. The two ovine β-defensin genes were first characterised in 1998 (Huttner *et al*, 1998) and named SBD1 and 2 (sheep beta defensin 1 and 2). SBD1 is expressed extensively in ovine epithelial tissues, including the trachea, tongue, rumen, reticulum, omasum and colon. In contrast, SBD2 is expressed in only the ileum and colon. Both peptides are regulated pre- and post-natally (Huttner *et al*, 1998).

2.6 MECHANISM OF ACTION OF ANIMAL ANTIMICROBIAL PEPTIDES

The second objective of the research presented in this thesis was to investigate the mechanism of action of ovine antimicrobial peptides. The mechanisms of action of antimicrobial peptides

are not fully understood. The mechanisms used seem to vary between peptides, and in some cases a single peptide can use different mechanisms depending on the organism it is confronted with. Numerous techniques have been developed to investigate the mechanisms of antimicrobial peptides, especially in relation to the interactions of the antimicrobial peptides with bacterial membranes. The most commonly used of these techniques were explored.

The current knowledge regarding the mechanism of action of animal antimicrobial peptides is also reviewed. There are several theories about the mechanisms used by antimicrobial peptides, so each of these is critically examined and compared. The known details of the mechanism of action of specific animal antimicrobial peptides are also described.

Finally, the previous work carried out to determine the mechanism of action of antimicrobial peptides from ovine blood is reviewed. From this, areas where knowledge is lacking are identified, so that the specific objectives of the current research project could be formulated.

2.6.1 Techniques to determine mechanisms of action of antimicrobial peptides

A number of assays have been developed to investigate the mechanism of action of antimicrobial peptides. These assays look at each proposed step in the mechanism of action individually.

To test whether antimicrobial peptides are able to bind to the outer surface of Gram-negative bacteria, the dansyl polymyxin B displacement assay is commonly used (Moore *et al*, 1986; Sawyer *et al*, 1988). Dansyl polymyxin B binds to the divalent-cation-binding sites of LPS, which results in enhanced fluorescence of the dansyl group. The affinity of antimicrobial peptides for LPS can be determined by measuring their ability to reduce the fluorescence by displacing the dansyl polymyxin B.

To test whether antimicrobial peptides are able to increase the permeability of the outer membranes of Gram-negative bacteria a few different assays have been used. These assays monitor the passage of a normally excluded molecule across the outer membrane. One assay uses lysozyme, which once taken up causes lysis of the cells (Hancock *et al*, 1981; Fidai *et al*, 1997). Another assay used 1-*N*-phenyl-napthylamine (NPN), which fluoresces only when it is in a hydrophobic environment (Loh *et al*, 1984; Patrzykat *et al*, 2002).

To study the interactions of antimicrobial peptides with the cytoplasmic membrane, model membranes are often used. Planar bilayers are used to measure the increases in conductance

of the ions (Gazit *et al*, 1996), liposomes are used to monitor the movement of fluorescent dyes (Zhang *et al*, 2000), and Langmir monolayers are used to measure the surface pressure (Zhang *et al*, 2000). These techniques can be used to investigate the effects of different lipid compositions on the activity of the peptides (Hristova *et al*, 1997).

Other studies investigate the interactions of antimicrobial peptides with the cytoplasmic membrane using intact bacteria. Ones method monitors the hydrolysis of the substrate *o*-nitrophenyl galactoside by the cytoplasmic enzyme β-galactosidase. This compound is usually excluded by the cytoplasmic membrane. Another method uses a florescent dye called 3,3-dipropylthiacarbocyanine ($DiSC_35$), which does not fluoresce when bound to the cytoplasmic membrane. However, it is released from the membrane and fluoresces when the membrane potential is reduced due to disruption (Wu and Hancock, 1999b; Zhang *et al*, 2000).

To study the interactions between antimicrobial peptides and inner cellular contents a few different tests are used. The ability of antimicrobial peptides to inhibit DNA, RNA and protein synthesis is commonly studied by monitoring the cells' incorporation of the radioactive precursors [*methyl*-^{3}H]thymidine, [5-^{3}H]uridine and L-[2,5-^{3}H]histidine, respectively (Patrzykat *et al*, 2002). The ability of the antimicrobial peptides to bind to DNA is determined by investigating the migration of peptide-treated DNA on an agarose gel (Park *et al*, 1998). Peptides with a high affinity for DNA inhibit its migration through the gel at low ratios.

2.6.2 Mechanisms of action

The interactions between animal antimicrobial peptides and microbial cells are not fully understood, but a number of models have been proposed. In all cases the interactions between the peptide and the membranes of the target cells play a key role.

The initial binding of the peptides to the outer cellular surface relies on the electrostatic interactions between the positively charged peptides and the negatively charged molecules on the surface of the cells (Ladokhin and White, 2001). The selectivity of the peptides for prokaryotic cells depends on the lipid composition associated with the membranes of these cells compared to those of eukaryotic cells. Gram-negative bacterial membranes contain LPS and Gram-positive bacterial membranes contain acidic polysaccharides, so in both cases the membranes are negatively charged. In contrast, animal cells are composed of predominately

zwitterionic and sphingomyelin phospholipids, to which antimicrobial peptides have a lower affinity (Oren and Shai, 1998).

In the case of Gram-negative bacteria, once the peptides have bound to the outer surface of the cells, they need to pass through this membrane in order to interact with the cytoplasmic membrane. When antimicrobial peptides bind to the divalent-cation-binding sites on LPS, they displace the native cations. This causes a distortion in the outer membrane structure, which increases the permeability of the membrane. The peptides are then able to pass through the outer membrane. This process is termed "self-promoted uptake" (Fidai *et al*, 1997)

Once the peptides pass through the outer membrane, they interact with the cytoplasmic membrane. This can lead to loss of cytoplasmic membrane function, including breakdown in membrane potential, loss of metabolites and ions, and alteration of membrane permeability. Three main mechanisms for the membrane permeation have been suggested (Bals and Wilson, 2003). These are illustrated in Figure 2.18.

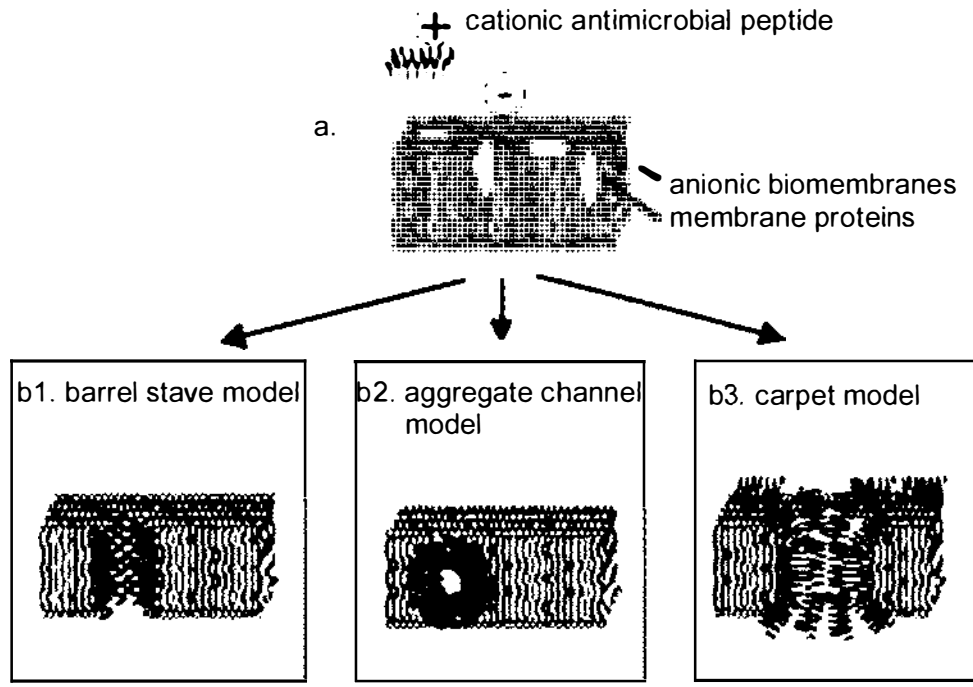

Figure 2.18 - Schematic diagram of the proposed mechanisms of permeability change of cytoplasmic membranes caused by antimicrobial peptides. After electrostatic interactions between the negatively charged bacterial wall and the positively charged peptides (a), the peptides may destabilise the membrane leading to cell death. Three models for this membrane destabilisation have been proposed; the barrel slave model (b1), the aggregate channel model (b2) and the carpet model (b3). This figure is adapted from that given by Bals and Wilson, 2000.

The first suggested mechanism of cytoplasmic membrane permeation is the barrel and stave model. This model involves the formation of voltage-dependent, transmembrane channels. The non-polar domains of the molecules face the membrane lipids to create a hydrophilic pore that spans the membrane. The barrel and stave mechanism involves four main steps: (1) binding of the monomers to the membrane in an amphipathic structure, (2) molecular recognition between membrane-bound peptide monomers that leads to their assembly, (3) insertion of at least two assembled monomers into the membrane to initiate the formation of a pore, and (4) progressive recruitment of additional monomers to increase the pore size (Oren and Shai, 1998). There is evidence for this mechanism being used by defensins (Kagan *et al*, 1990; Wimley *et al*, 1994).

The second suggested mechanism of cytoplasmic membrane permeation is the aggregate channel model. This model involves the arrangement of the peptides in unstructured clusters in the membrane, which allows the dynamic formation of pores for short periods of time. This model is most likely used by peptides that are too small to span the membrane (Fidai *et al*, 1997). This can lead to intracellular components leaking out and the peptides entering the intracellular space. Antimicrobial peptides that pass through the cytoplasmic membrane and interact with the inner cellular contents include the dodecapeptide bactenecin (Wu and Hancock, 1999b), the proline/arginine-rich peptides PR-39 (Boman *et al*, 1993; Cabiaux *et al*, 1994), the two bovine cathelicidins Bac5 and Bac7 (Skerlavaj *et al*, 1990) and the tryptophan-rich indolicidin (Subbalakshmi and Sitaram, 1998; Friedrich *et al*, 2001).

The third suggested mechanism of cytoplasmic membrane permeation is the carpet model. This involves the covering of the microbial cells with the peptide. The integrity of the membrane collapses via holes that form by the bending of the lipid bilayer back on itself. There are four proposed steps in this model: (1) preferential binding of positively charged peptide monomers to the negatively charged phospholipids, (2) laying of amphipathic monomers on the surface of the membrane so that the positive charges of the basic amino acids interact with the negatively charged phospholipid headgroups or water molecules, (3) rotation of the molecule leading to reorientation of the hydrophobic residues towards the hydrophobic core of the membrane, and (4) disintegrating the membrane by disrupting the bilayer curvature leading to micellisation (Oren and Shai, 1998). The mechanism is used by α-helical peptides such as the insect cecropins, the amphibian magainins and dermaseptins

(Oren and Shai, 1998) and the cathelicidins LL-37 (Oren *et al*, 1999) and SMAP29 (Schuster and Jacob, 1992).

2.6.3 Mechanism of action of ovine antimicrobial peptides

Of the seven predicted ovine cathelicidins that may be present in ovine neutrophils, only SMAP29, and peptides based on SMAP29, have been synthesised and studied in regard to their mechanism of action. An investigation into the mechanisms of action of a derivative of SMAP29 (residues 1 to 18) against yeast showed that the peptide disrupted the structure of the cell membrane by interacting with the lipid bilayer (Schuster and Jacob, 1992). Another study showed that ovisprin, an 18-residue peptide based on SMAP29, orientates parallel to the lipid bilayer. This is consistent with the carpet mechanisms of membrane disruption, which is common for α-helical peptides. However, in contrast, immunoelectron microscopy showed that SMAP29 permeated the outer and inner membranes of *E. coli* cells almost immediately, and then entered the bacterial cytoplasm (Kalfa *et al*, 2001). Therefore, the target of SMAP29 is not fully understood and may be different in different circumstances.

The mechanisms of action of the other predicted ovine cathelicidins have not been investigated. However, it is likely that they would behave similarly to the bovine proline/arginine-rich cathelicidins and bovine bactenecin. This means that the other ovine cathelicidins probably pass through the inner cellular membrane and interact with the inner cellular contents.

2.7 MORPHOLOGICAL CHANGES TO MICROBIAL CELLS INDUCED BY ANIMAL ANTIMICROBIAL PEPTIDES

The third objective of the research presented in this thesis was to examine the morphological changes to bacterial cells induced by ovine antimicrobial peptides, in order to further understand the mechanism of action of these peptides. Previously, two microscopy techniques have been commonly used to investigate morphological changes induced by antimicrobial peptides. These are transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The advantages and disadvantages of each of these techniques are investigated. As well as this, the potential for a relatively new technique, atomic force microscopy (AFM), to be useful for this application is also examined.

The knowledge gathered about bacterial cell morphological changes induced by antimicrobial peptides is also reviewed. Like the mechanism of action studies these results were often contradictory which again implies that antimicrobial peptides may have different effects depending on the microbial cells being attacked and the environmental conditions.

Finally, the previous work carried out to determine the effect of ovine antimicrobial peptides of the morphology of microbial cells is reviewed. From this, areas where knowledge was lacking were identified, so that the specific objectives of the current research project could be formulated.

2.7.1 Techniques to investigate morphological changes

To investigate the morphological changes to microbial cells induced by antimicrobial peptides, two microscopy techniques, TEM and SEM, are commonly used. These techniques are complementary because TEM looks at a cross-section of the sample, and SEM looks at the surface of the sample.

Both TEM and SEM use an electron microscope to image the samples. An electron gun produces a stream of monochromatic electrons. This stream is focused to a small, thin line by the two condenser lenses, and then the condenser aperture, which removes the high angle electrons, restricts the beam. The beam strikes the sample and then parts of the beam are transmitted. The transmitted beam is focused by the objective lens and further restricted by the objective and selected area apertures. The image is enlarged by the intermediate and projector lens, before it strikes the phosphor image screen and generates light. The darker areas of the image represent those areas of the sample that fewer electrons were transmitted through and the lighter areas represent those areas of the sample that more electrons were transmitted through.

Another technique, which has not previously been used to image antimicrobial peptide-treated cells, but may be useful, is AFM. Like SEM, AFM gives an image of the surface of the sample. The advantage of AFM is that it can be used to image cell changes in real-time (Ahimou *et al*, 2003). This is not possible with SEM because the cells are fixed prior to imaging. AFM is also simpler, more convenient and less costly than SEM, which requires an ultravacuum environment (Amro *et al*, 2000).

AFM uses a cantilever with a sharp tip to probe the surface of the sample (Prater *et al*,). The cantilever is between 100 and 200μm long, and the tip is approximately 2μm long and less than 100Å in diameter. When the tip and the sample come into close proximity, the force between the two causes the cantilever to bend or deflect. As the tip scans over the sample the movement in the cantilever is detected by using a laser beam that bounces off the sample onto a position-sensitive photodetector. This information is used to generate a map of the surface topography of the sample.

The use of AFM to image living cells is relatively new (Dufrene, 2002). It has been successfully used to image live bacteria (Boonaert *et al*, 2000), fungi (Kasas and Ikai, 1995; Gad and Ikai, 1995; Boonaert *et al*, 2000; Ahimou *et al*, 2003) and animal cells (Kasas *et al*, 1993). AFM has also been used to image *Bacillus subtilis* treated with penicillin (Kasas *et al*, 1994) and *E. coli* treated with cefodizime (Braga and Ricci, 1998).

2.7.2 Morphological changes

Microscopy techniques are commonly used to confirm theories and gain further knowledge about the mechanism of action of antimicrobial peptides. For defensins and α-helical peptides, the target is thought to be the cytoplasmic membrane of bacterial cells, so it is expected that these peptides would induce notable differences in the morphology of bacterial cells. Numerous TEM and SEM studies have confirmed that defensins cause damage to the cytoplasmic membranes of both Gram-negative (Sawyer *et al*, 1988; Eisenhauer *et al*, 1989) and Gram positive bacteria (Shimoda *et al*, 1995; Miyakawa *et al*, 1996). Similarly, α-helical peptides also cause visible damage to the cytoplasmic membrane (Tiozzo *et al*, 1998; Oren *et al*, 1999).

Similarly, for other antimicrobial peptides the target is thought to be the inner cellular contents, not the cytoplasmic membrane, so it is expected that these cells would not induce damage to the bacterial membranes. Studies have shown that indolicidin-treated *E. coli* cells (Subbalakshmi and Sitaram, 1998) and PR-39-treated *S. typhimurium* (Shi *et al*, 1996a) became filamentous, which may have been induced by inhibition of DNA synthesis.

2.7.3 Morphological changes induced by ovine antimicrobial peptides

Like the mechanism of action studies, the only ovine antimicrobial peptide that has been studied for its induced morphological changes is SMAP29. SEM showed SMAP29 rapidly

caused alterations, including blebs and blisters, to the surface of both Gram-negative and Gram-positive bacteria (Skerlavaj *et al*, 1999; Saiman *et al*, 2001; Arzese *et al*, 2003). This is consistent with the idea that SMAP29 acts on the cytoplasmic membrane of bacterial cells.

The morphological changes to bacterial cells induced by other ovine cathelicidins have not been investigated. However, since the majority of these peptides are proline/arginine-rich, like porcine PR-39, it is expected that these peptides would also cause the bacteria cells to become filamentous.

2.8 EFFECT OF ENVIRONMENTAL CONDITIONS ON ACTIVITY OF ANIMAL ANTIMICROBIAL PEPTIDES

The fourth objective of the research presented in this thesis was to investigate the effects of environmental conditions on the activity of ovine antimicrobial peptides. If the antimicrobial peptides isolated from ovine blood are to be used as therapeutics agents or biopreservatives, as discussed in Section 2.4.2, then they will need to be active in a variety of environmental conditions. Therefore, techniques for testing factors that may influence the antimicrobial activity of the peptides are investigated.

The current knowledge regarding the effects of various environmental conditions on antimicrobial peptides activity is also reviewed. This includes the effects of physical factors such as pH, monovalent and divalent cation concentration and temperature. The effects of using the antimicrobial peptides in combination compared to alone was also researched.

Finally, the previous work carried out to determine the effect of environmental conditions on the antimicrobial activity of ovine antimicrobial peptides was reviewed. From this, areas where knowledge was lacking were identified, so that the specific objectives of the current research project could be formulated.

2.8.1 Techniques to determine effect of environmental conditions

In order to investigate the effect of environmental conditions on the activity of antimicrobial peptides, a few different techniques have been used. Environmental factors that have been tested for their effects include salt concentration, cation concentration, pH and temperature. The antimicrobial activity of the peptides has also been examined when they are used in combination with each other, or with other antimicrobial compounds (Selsted *et al*, 1985).

To test the effect of environmental conditions on antimicrobial peptide activity the growth media are usually altered to the required test conditions. In most studies the MIC of the peptides in different media is determined using either the diffusion plate assay (Harwig *et al*, 1995; Cho *et al*, 1998), or the micro-broth dilution method (Scott *et al*, 1999). In other studies, the log decrease in viable cells (Selsted *et al*, 1985), or the percentage by which the peptide is inhibited is measured (Lehrer *et al*, 1983).

To test the effect of antimicrobial peptides in combination with each other or with known antimicrobial substances the fraction inhibitory concentration (FIC) has been used (Fidai *et al*, 1997; Scott *et al*, 1999). This method is similar to the micro-broth dilution MIC method, except that it uses checkerboard titrations, where one compound is diluted down the columns and the other is diluted across the rows, in a microtitre plate. The FICs are calculated using the formula: FIC = $[A]/MIC_A + [B]/MIC_B$. MIC_A and MIC_B are the MICs of the compounds A and B alone. [A] and [B] are the MICs of compounds A and B when in combination. An FIC of less than 0.5 means the compounds are synergistic, an FIC of unity means the compounds are additive, and an FIC of greater than four means that the peptides are antagonistic.

2.8.2 Effects of environmental conditions

The most commonly tested factor for its effect on antimicrobial activity is salt concentration. At a physiological NaCl concentration of 100mM, defensins (Lehrer *et al*, 1983), (Selsted *et al*, 1985), (Aley *et al*, 1994), and some cathelicidins, such as prophenin (Harwig *et al*, 1995), are considerably inhibited. In contrast, other antimicrobial peptides, such as the α-helical cathelicidins (Travis *et al*, 2000; Zhao *et al*, 2001), and the proline/arginine-rich cathelicidins (Shamova *et al*, 1999), are not inhibited in these conditions.

As well as the effect of Na^+, the effect of other cations, particularly the divalent cations Mg^{2+} and Ca^{2+}, have been investigated. All animal antimicrobial peptides tested are inhibited considerably by divalent cations. This is because these peptides bind to the cation-binding sites of LPS on the surface of Gram-negative bacteria so the peptides have to compete with the ions. In most cases, Ca^{2+} ions are more inhibitory than Mg^{2+} ions (Selsted *et al*, 1985; Aley *et al*, 1994; Turner *et al*, 1998). This may be due to Ca^{2+} being a larger ion than Mg^{2+}. However, in one study Mg^{2+} ions were more inhibitory than Ca^{2+} ions (Lawyer *et al*, 1996). It was also shown that the effect of cations is accumulative so it is the overall cation

concentration that is important, not the concentration of the individual cations (Cho *et al*, 1998).

Another factor that has been investigated for its effect on antimicrobial activity is pH. The antimicrobial peptides tested all have decreased antimicrobial activity in acidic pH conditions compared to neutral and slightly basic pH conditions (Lehrer *et al*, 1983). The human cathelicidin LL-37 has an α-helical structure at neutral and basic pH values but not at acidic pH values (Johansson *et al*, 1998). This conformational change is probably brought about by the acidic side chains being protonated at low pH values and it is this conformational change that is probably responsible for the peptide's change in activity.

The effect of temperature on the activity of antimicrobial peptides has not been investigated in detail. It is known that SMAP29 retains its activity after heating to 65°C (Mahoney *et al*, 1995); however, other peptides have not been tested.

The effect of antimicrobial peptides in combination with each other and with known antimicrobial substances is another area that has been studied. Cathelicidin peptides are synergistic, but only in some combinations (Yan and Hancock, 2001). The amphibian peptide magainin II is synergistic with β-lactams (Scott *et al*, 1999).

2.8.3 Effects of environmental conditions on ovine antimicrobial peptides

Only a few studies have been carried out to investigate the effects of conditions on ovine antimicrobial peptides. It has been shown that the predicted ovine α-helical peptides SMAP29 and SMAP34 (Travis *et al*, 2000) and the purified ovine proline/arginine-rich peptide OaBac5α (Shamova *et al*, 1999) are not significantly impaired by high (100mM) NaCl concentrations. As previously mentioned SMAP29 retains its antimicrobial activity after being heated to 65°C for 30 minutes (Mahoney *et al*, 1995).

The effects of other factors on the activity of ovine antimicrobial peptides, such as cation concentration and pH, have not been investigated. The activity of ovine antimicrobial peptides in combination with each other or with other antimicrobial substances has also not been examined.

2.9 PILOT-SCALE EXTRACTION OF ANIMAL ANTIMICROBIAL PEPTIDES

The final objective of the research presented in this thesis was to determine whether it was possible to extract antimicrobial peptides from ovine blood on a scale larger than that used in the laboratory. No studies have previously been reported on the large-scale extraction of antimicrobial peptides from blood. This is probably because, as discussed in Section 2.4.1, most research groups focus on applications of synthetic antimicrobial peptides, not those that can be purified from natural sources.

However, animal blood is routinely processed commercially (Ockerman and Hansen, 1988; van Asch, 2001). Various products are isolated from blood plasma including serum albumins, fibronectin, transferrins and trypsin; and the red blood cells including haemoglobin protein. Centrifugation and ultrafiltration are often used in commercial operations to remove some of these fractions. A similar approach may be feasible for the isolation and fractionation of proteins from white blood cells.

2.10 CONCLUSIONS

The first objective of this research project was to purify and characterise the antimicrobial substances found in ovine blood. This literature review showed that ovine neutrophils are predicted to contain seven cathelicidin peptides. Of these antimicrobial peptides, variants of only one have been previously purified. Therefore, the aim of this research was to purify and characterise the other antimicrobial peptides from ovine blood, as well as other components which have antimicrobial activity.

The second objective of this research project was to determine the mechanism of action of ovine antimicrobial peptides. This literature review showed that antimicrobial peptides can have a number of different mechanisms of action depending on their structures. Studies with ovine SMAP29 gave contradictory results so the mechanism of action of this peptide is unclear. The mechanisms of action of the other ovine cathelicidins have not been investigated. It was hypothesised that the proline/arginine-rich cathelicidins will interact with the inner cellular contents of the bacterial cells, whereas the α-helical peptides will target the cytoplasmic membrane. Therefore, the aim of this research was to investigate and compare the mechanisms of action of ovine proline/arginine-rich peptides and α-helical peptides.

The third objective of this research project was to investigate the morphological changes to bacterial cells induced by ovine antimicrobial peptides. This literature review showed that antimicrobial peptides induce different morphological changes to microbial cells depending on their mechanism of action. Studies with ovine SMAP29 showed that it damages the cell membranes. The morphological changes to microbial cells induced by the other ovine cathelicidins have not been investigated. It was hypothesised that the proline/arginine-rich cathelicidins would not damage the membranes of the bacterial cells, whereas the α-helical peptides would target the cytoplasmic membrane. Therefore, the aim of this research was to investigate and compare the morphological changes to bacterial cells induced by ovine proline/arginine-rich and α-helical peptides.

The fourth objective of this research project was to determine the effect of different environmental factors on the activity of ovine antimicrobial peptides. This literature review showed that some antimicrobial peptides are inhibited by high salt-concentrations, divalent cations, and acidic pH values; whereas other peptides are not. Studies with ovine SMAP29 showed it was not impaired by high salt concentrations or by heating to 65°C. Other conditions have not been tested. The effects of environmental factors on the other ovine cathelicidins have also not been investigated. Therefore, the aim of this research was to determine the effects of various factors on the activity of ovine proline/arginine-rich and α-helical peptides.

The final objective of this research project was to determine whether it is possible to produce an active antimicrobial crude extract on a larger scale than in the laboratory using industrial-style equipment. This literature review showed that antimicrobial peptides have not been extracted from animal blood in large quantities before. Blood is routinely fractionated on a commercial scale to isolate various high-value proteins. Therefore, the aim of this research was to determine whether antimicrobial peptides can be extracted from ovine blood on a large scale and to develop a process that could be easily operated industrially.

CHAPTER 3
MATERIALS AND METHODS

3.1 MATERIALS AND METHODS USED FOR PEPTIDE PURIFICATION

3.1.1 Crude extraction

Antimicrobial peptides were extracted from neutrophil granules using a process adapted from methods given in the literature (Eisenhauer *et al*, 1989; Borenstein *et al*, 1991; Selsted *et al*, 1993). The pooled blood from a number of lambs was collected from Feilding Lamb Packers (Feilding, New Zealand), mixed with 10% (w/v) aqueous sodium citrate in a ratio of 10:1 to stop coagulation, and stored on ice until used. Before processing, the blood was filtered to remove any solid contaminants such as wool and clotted blood.

Two different methods were used to lyse the red blood cells. The original process was based on that used by Selsted's group at the University of California, Irvine (Selsted *et al*, 1993). The blood cells were separated from the blood plasma by centrifugation. The red blood cells were then lysed by adding water (3:1 v/v) and mixing for ten seconds, before triple-strength PBSX buffer (411mM NaCl, 8.1mM KCl, 1.5mM $MgCl_2$, 24.3mM Na_2HPO_4 and 4.5mM KH_2PO_4 (pH 7.4)) was added to restore the ionic balance. The white blood cells were collected by centrifugation. Usually it was necessary to repeat the red blood cell lysis steps at least once to ensure all the red blood cells were removed.

Because this extraction process was time-consuming and used large amounts of buffer, it was replaced by one based on that of Robert Lehrer's group at the University of California, Los Angeles (Borenstein *et al*, 1991). In this process the blood cells were not separated from the plasma before the red blood cells were lysed. This reduced the amount of centrifugation required. To lyse the red blood cells, 0.83% (w/v) aqueous ammonium chloride solution was used instead of water. The ammonium chloride solution was mixed with whole blood (2:1 v/v). This was advantageous because it was not necessary to add buffer to stop white blood cell lysis because the white blood cells were relatively stable in the ammonium chloride solution. The red blood cell lysis was also more effective than the previous method, so it was usually not necessary to repeat the lysis steps.

After the red blood cell lysis step, the white blood cells were collected by centrifugation (Sorvall centrifuge, SS-3 rotor, 700g, 15 minutes, 4°C) and resuspended in PBSX buffer (137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4). The cells were stained using a Diff-Quick staining kit (Baxter Cat. No. B4132-1) and then examined under a microscope to see if any intact red blood cells remained. If present, the ammonium chloride lysis step was repeated.

The white blood cells were disrupted using sonication (MSE sonicator, three times for 30 seconds at 8 microns peak to peak) to release the neutrophil granules. These granules were collected by high-speed centrifugation (Sorvall centrifuge, SS-34 rotor, 27,000g, 40 minutes, 4°C). Again, the cells were stained with the Diff-Quick staining kit and examined under the microscope. If intact white blood cells were still present, sonication was repeated.

The antimicrobial peptides were extracted from the granules in 10% acetic acid with stirring overnight at 4°C. The solution containing the peptides was separated from the granules (Sorvall centrifuge, SS-34 rotor, 27,000g, 20 minutes, 4°C). This solution was rotary – evaporated (bath temperature 35-40°C) and freeze-dried. The extracted solids were then suspended in 0.01% acetic acid. This solution is referred to as the crude extract.

3.1.2 Gel electrophoresis

SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) was used to determine the number and approximate size of the proteins and peptides present in the crude extract and the gel filtration fractions. To separate peptides and proteins 22% and 15% acrylamide gels were used, respectively. The peptide gel contained 2.5mL 1.5mM Tris-HCl buffer, 100µL 10% SDS stock, 7.32mL 30% acrylamide stock (30g acrylamide and 0.8g methlenebis acrylamide per 100mL distilled water), 5µL N,N,N',N'-tetramethylethylenediamine (TEMED) and 50µL 10% ammonium persulphate solution. The protein gel contained 2.02mL distilled water, 2.5mL 1.5mM Tris-HCl buffer, 100µL SDS stock, 5.3mL 30% acrylamide stock, 5µL TEMED and 50µL 10% ammonium persulphate solution. In both cases the separating gels were covered with a stacking gel to increase the resolution. The stacking gel contained 6.1mL distilled water, 2.5mL 0.5mM Tris-HCl buffer, 100µL SDS stock, 1.3mL 30% acrylamide stock, 10µL TEMED and 50µL 10% ammonium persulphate solution.

The samples were diluted with an equal volume of double-strength sample buffer (25mL 0.5M Tris-HCl buffer, 20mL glycerol, 40mL 10% SDS, 10mL β-mercaptoethanol and 5mL 0.05% bromophenol blue) and heated at 80°C for three minutes, before they were added to the gels. The gels were run at 20mA per gel for 45 minutes (Hoefer Scientific Instruments, Mighty Small II, SE 250) with 200mL running buffer (3g Tris, 14.5g glycine and 1g SDS in 1L of distilled water).

The gel was fixed for one hour (25% isopropanol and 10% acetic acid in distilled water), stained for two hours (1.25g Coomassie Brilliant Blue R250 to 242mL distilled water, 242mL methanol and 46mL acetic acid) and destained overnight (7.5% acetic acid and 5% methanol in distilled water). The gels were covered in GelAir Cellophane Support (BioRad), which allowed them to dry without cracking. Images of the gels were captured using a digital scanner.

3.1.3 Gel filtration

Gel filtration was used to separate the components in the crude neutrophil extract according to their sizes. The extract was passed through a column packed with Bio-Gel P10 (Bio-Rad Laboratories, California). The running buffer (5% acetic acid) was pumped through the column at a flow rate of 20mL/hour. The eluant was passed through a UV detector (LKB Bromma 2138 UVICORD S), and the absorbance was plotted over time using a chart recorder (Sekonic SS-250F). A fraction collector (LKB Ultrorac 7000) was used to collect the eluant in 10-minute fractions. The chart recorder was run at a speed of 30mm/hour so that 5mm on the chromatograph chart corresponded to one fraction. The fractions that constituted each peak on the chromatograph were pooled, freeze-dried and dissolved in 0.01% acetic acid.

3.1.4 Cationic-exchange chromatography

As an alternative to gel filtration, cationic-exchange chromatography was used to separate the cationic molecules from the other components present in the crude extract. The extract was passed through a Macro-Prep CM column (BioRad Laboratories, California). The cationic compounds bound to the anionic resin, and the non-cationic molecules were washed through with 25mM ammonium acetate. The cationic molecules were eluted with 10% acetic acid, freeze-dried and dissolved in 0.01% acetic acid. This system was set-up in the same way as the gel filtration system already described (Section 3.1.3).

3.1.5 Peptide purification using HPLC

RP-HPLC (reverse-phase high performance liquid chromatography) was used to separate the components in the active gel filtration and cation exchange fractions. A Waters HPLC system with a Phenomenex Jupiter C-18 column was used to separate the components in the active gel filtration fraction. A Dionex HPLC system with a Phenomenex Jupiter Proteo column was used to separate the components in the cationic fraction. In both cases the peptides were separated using an acetonitrile gradient. Buffer A was 5% acetonitrile with 0.1% TFA (trifluoroacetic acid), and Buffer B was 95% acetonitrile with 0.1% TFA. The elution of the peptides was monitored using a UV detector (230nm for the Waters system and 215nm for the Dionex system). The peptide fractions were collected manually, freeze-dried, and dissolved in 0.01% acetic acid.

3.1.6 Radial diffusion plate assay

The radial diffusion plate assay method (Steinberg and Lehrer, 1997) was used to test if the crude extract, gel filtration fractions, ion-exchange fractions, and HPLC peaks displayed antimicrobial activity. The test cultures used were *Escherichia coli* O111 (Gram-negative bacterium), *Staphylococcus aureus* NCTC 4163 (Gram-positive bacterium) and *Candida albicans* 3153A (yeast). These cultures were sourced from the Massey Microbiology Department, the NCTC (National Collection of Type Cultures) and the NCPF (National Collection of Pathogenic Fungi), respectively.

The culture was mixed with the underlay agar (10mL trypticase soy broth, 10g ultrapure agarose (Sigma A-6013), 1L distilled water, pH 7.4), which contained limited nutrients, and was allowed to set. Wells were made in the agar and 5µL of the test sample was added to each well. For each plate both a positive and a negative control were included. The positive control was a common antibiotic; polymyxin B for Gram-negative bacteria, nisin for Gram-positive bacteria and nystatin for yeast. The negative control was 0.01% acetic acid because this was used to dissolve the samples. The plates were left for one hour to allow the test solutions to diffuse into the agar. After this, the overlay agar (60g trypticase soy broth powder, 10g ultrapure agarose, 1L distilled water), which was rich in nutrients, was added. The plates were incubated overnight at 37°C. The following morning the diameters of the clearings around the wells were recorded.

3.1.7 Radial diffusion plate assay MIC method

The radial diffusion plate assay method was used to determine the minimum inhibitory concentrations (MICs) of the purified peptides (Lehrer *et al*, 1991). This method was based on the radial diffusion plate assay method given in Section 3.1.6 but multiple dilutions of each sample were tested. The MICs were also determined in the presence of salt. This was achieved by adding 100mM NaCl to the underlay agar

The diameters of the cleared zones were recorded for each peptide concentration. The assay was carried out only once for each peptide due to the limited amount of purified material available. The log of the peptide concentration was plotted against the diameter of the clearing on the plate (less the size of the well), and a straight line was fitted. Linear regression analysis was carried out using the statistical package GenStat. The "simple linear regression" function, which uses the least-squares method, was used. The Ln peptide concentration was the explanatory variant (x-axis variant) and the clearing size was the response variant (y-axis variant). From the GenStat outputs the gradient and y-intercept were recorded. From this the MICs were determined by calculating the point at which the line crossed the x-axis. This was the peptide concentration required to make a clearing size of zero. The limits of the 95% confidence intervals for the x-intercept were calculated using Equation 3.1 (Draper and Smith, 1981).

$$\text{Limits of confidence intervals} = g(\hat{X}_0 - \bar{X}) \pm \frac{t}{b_1}\sqrt{s^2}\sqrt{\frac{(\hat{X}_0 - \bar{X})}{Sxx} + \frac{(1-g)}{n}} \qquad \textbf{Equation 3.1}$$

where:

$g = \text{constant} = \dfrac{t^2 s^2}{b_1 Sxx}$

$\hat{X}_0$ = the estimated x-intercept

$\bar{X}$ = the mean of the x values

t = the t-value

b_1 = gradient of the line-of-best-fit

s^2 = estimate of the pooled variance

Sxx = total corrected sum of the squares for $x = (X_1 - \bar{X})^2 + (X_2 - \bar{X})^2 + (X_3 - \bar{X})^2 + (X_4 - \bar{X})^2$

n = the number of x values

The relationship between the bounds of the confidence intervals and the line-of-best-fit is demonstrated in Figure 3.1. The upper limit of the x-intercept is a lower value than the lower

limit. In some cases the limits of the 95% confidence intervals of the x-intercept could not be calculated. This was because the bounds of the confidence intervals sometimes did not cross the x-axis. An example of this is illustrated in Figure 3.2

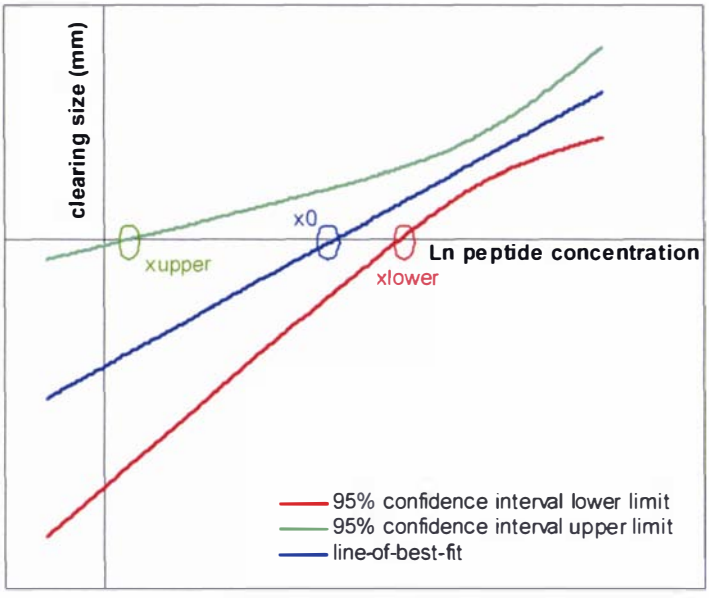

Figure 3.1 - Graph of Ln peptide concentration versus clearing size showing the relationship between the line-of-best-fit and the bounds of the 95% confidence intervals for the line.

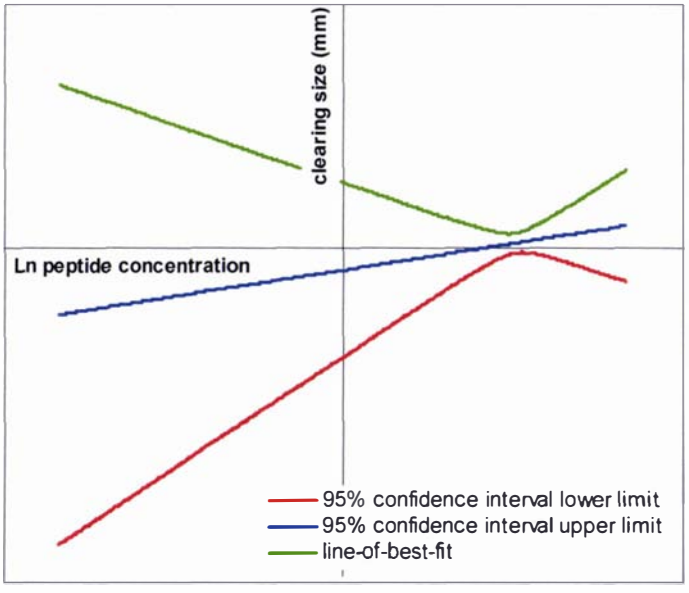

Figure 3.2 - Graph of Ln peptide concentration versus clearing size showing the relationship between the line-of-best-fit and the bounds of the 95% confidence intervals for the line when the bounds do not cross the x-axis.

3.1.8 Mass spectroscopy

Mass spectroscopy was used to determine the molecular weight of the purified peptides. Mass spectroscopy was carried out at the Proteome Analysis Facility, Sydney, Australia, and

the School of Biological Sciences, University of Auckland, New Zealand. At the Proteome Analysis Facility a Micromass Q-TOF-MS equipped with a nanospray source was used. The data were manually acquired using borosilicate capillaries over the m/z range 400-16000Da. At the School of Biological Sciences a Voyager DE PRO MALDI-TOF mass spectrometer from Applied Biosystems was used. The ion-acceleration potential used was 20 kv, and for each sample the "100 shots" mode was used to acquire data within the 500-8000Da range of positive polarity.

3.1.9 N-terminal sequencing

N-terminal sequencing was used to determine part of the amino acid sequence of the purified peptides. The N-terminal sequencing was carried out at the Proteome Analysis Facility, Sydney, Australia, and the School of Biological Sciences, University of Auckland, New Zealand. At both facilities automated Edman degradation using an Applied Biosystems Procise Protein Sequencing System was used. In the Edman degradation the N-terminal residue was labelled and cleaved from the peptide. This residue was identified by its retention time using RP-HPLC. The procedure was repeated to identify the next amino acid, and so on for subsequent amino acids.

3.1.10 Peptide characterisation

Online bioinformatics tools were used to characterise the purified peptides by comparing them to other known peptides and proteins. The theoretical molecular weights of the antimicrobial peptides predicted from ovine cDNA were calculated using the ExPASy PeptideMass software (http://au.expasy.org/tools/peptide-mass.html). These masses were then compared to the experimentally determined molecular weights of the purified peptides.

For the purified cathelicidin peptides, the N-terminal sequences were manually compared to the sequences of the predicted peptides. When the purified peptides were determined to be truncated forms of the predicted cathelicidins, then the peptidase database MEROPS (http://merops.sanger.ac.uk/) was searched to identify peptidases that could have performed the truncation.

For the purified peptides with N-terminal sequences that did not match any of the cathelicidin sequences, the ExPASy BLAST software (http://au.expasy.org/tools/blast/) was used to compare the N-terminal sequences of the purified peptides with known proteins and peptides.

3.1.11 Analysis of proline/arginine-rich sequences

The sequences of all the known proline/arginine-rich cathelicidin peptides were compared to find similarities. The sequence motifs and repeats were detected manually. The hydrophobicities of the peptides were compared by generating graphs using the ExPASy ProScale Kyte/Doolittle hydrophobicity plot tool (http://au.expasy.org/tools/protscale.html). Similarly, the polarities of the peptides were compared by generating graphs using the ExPASy ProScale Zimmerman polarity plot tool (http://au.expasy.org/tools/protscale.html).

3.2 MATERIALS AND METHODS USED FOR MECHANISM OF ACTION TESTS

3.2.1 Peptide synthesis

Three peptides were synthesised based on the known ovine peptides. This was necessary because the time required to purify sufficient amounts of the pure peptides to carry out a large number of experiments was too long. Between 15 and 20mg of each peptide were produced. To do this, *N*-(9-fluorenyl)methoxy carbonyl solid-phase peptide synthesis using an Applied Biosystems model 432A synthesiser was carried out at the University of British Columbia Nucleic Acid/Protein Service facility. The purities of the peptides were confirmed to be at least 99% using high-performance liquid chromatography (HPLC) and mass spectroscopy analysis.

The sequences of the three peptides are given in Table 3.1. The full OaBac5 molecule could not be synthesised, due to the large number of proline residues, so a shortened version (the first 25 amino acids) called OaBac5mini was created. OaBac7.5mini was made in its natural form, the C-terminal 29 amino acids of OaBac7.5. SMAP29 was made as a 28 amino acid amidated peptide because this is thought to be its natural form (Skerlavaj *et al*, 1999).

Table 3.1 - Sequences of ovine antimicrobial peptides used for this research

Peptide	Sequence
OaBac5mini	RFRPPIRRPPIRPPFRPPFRPPVR-NH_2
OaBac7.5mini	RRIPRPILLPWRPPRPIPRPQPQPIPRWL-NH_2
SMAP29	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH_2

3.2.2 Micro-broth dilution MIC method

The minimum inhibitory concentrations (MICs) of the ovine-derived peptides against a variety of organisms were determined using a modified broth dilution method (Wu and Hancock, 1999a). This method is based on the standard micro-broth dilution method recommended by the National Committee of Laboratory Safety and Standards (NCLSS)(Amsterdam, 1996). The changes to the standard method included: the use of polypropylene plates instead of polystyrene plates - because the cationic peptides bind to polystyrene plates and are inactivated; and using Mueller-Hinton broth (MHB) instead of trypticase-soy broth (TSB) - because the activity of the peptides can be inhibited by the salt in TSB. The test organisms used included eight Gram-negative bacteria, five Gram-positive bacteria and two yeasts. The test organisms and there sources are listed in Table 3.2

Table 3.2 - Microorganisms used for micro-broth dilution minimum inhibitory concentration tests and their sources

Microorganism	Source
Escherichia coli O111	Communicable Disease Centre, New Zealand.
E. coli UB1005	Department of Microbiology and Immunology, University of British Columbia, Canada.
E. coli DC2	Department of Microbiology and Immunology, University of British Columbia, Canada.
E. coli O157:H7	Communicable Disease Centre, New Zealand.
Salmonella typhimurium 14028s	Department of Microbiology and Immunology, University of British Columbia, Canada.
S. typhimurium MS4252S	Department of Microbiology and Immunology, University of British Columbia, Canada.
Pseudomonas aeruginosa PAO1	Department of Microbiology and Immunology, University of British Columbia, Canada.
P. aeruginosa Z61	Department of Microbiology and Immunology, University of British Columbia, Canada.
Staphylococcus aureus NCTC 4163	National Type Culture Collection, England.
S. aureus MRSA R147	Department of Microbiology and Immunology, University of British Columbia, Canada.
S. aureus 1056 MRSA	Institute of Food, Nutrition and Human Health, Massey University, New Zealand.
S. epidermidis clinical isolate	University of British Columbia Children's Hospital, Canada.
Enterococcus faecalis ATCC 29212	American Type Culture Collection, USA.
Candida albicans 105	Department of Microbiology and Immunology, University of British Columbia, Canada.
C. albicans 3153A	Institute of Molecular Biosciences, Massey University, New Zealand.

For these assays, Sigma 96-well polypropylene microtitre plates were used. The peptides were diluted across the rows in two-fold serial dilutions from 64 to 0.0625μg/mL. Each well contained 50μL of peptide diluted in a solution containing 0.2% bovine serum albumin (BSA) and 0.01% acetic acid. To each well, 50μL Mueller-Hinton broth (Difco) containing $2x10^5$ cells was added, to give a final concentration of $1x10^5$ cells. The plates were incubated overnight at 37°C. The next day the wells that contained culture growth were recorded. The assessment was done visually. If the culture was turbid or contained a pellet, it was considered to have grown. The MIC was defined as the concentration of the peptide in the last well in which culture growth did not occur.

The assay was carried out five times independently. For each run duplicates of each sample were included. For each test organism in each run, a control peptide was also tested. The controls were polymyxin B for the Gram-negative bacteria, nisin for the Gram-positive bacteria and nystatin for the yeast. The MICs of these peptides were generally consistent across the runs (approximately 90% of the time they were the same).

The MICs were reported as the geometric means and the 95% confidence intervals for the means. The geometric mean was used, as opposed to the arithmetic mean, because the data sets were skewed instead of normally distributed. The use of the geometric mean meant that the larger values had less influence on the mean. The data values were log-transformed, and then the means, standard deviations and 95% confidence intervals of the log-transformed data were determined using the functions "AVERAGE", "STDEV" and "CONFIDENCE" in Microsoft Excel. The means and the 95% confidence limits were exponentially-transformed to give the geometric means and 95% confidence intervals of the means of each original data set. The variation was asymmetric because of the log-transformation.

3.2.3 Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was used to investigate the secondary structures of the peptides in different solutions. The CD spectra were collected using a Jasco J-810 spectropolarimeter connected to a Jasco spectra manager using a quartz cell with a path length of 1mm. The CD spectra were measured at 25°C, between 190 and 250 nm at a scanning speed of 50nm/minute. For each peptide, CD spectra were collected for 25μM peptide in 10 mM phosphate buffer (pH 7.2), 50% 2,2,2-trifluroethanol (TFE), and 10mM lyso-PC/lyso-PG (1:1). For each solution ten scans were carried out and averaged. Because the peptide

solutions were dynamic each scan was slightly different. The CD spectra of the solutions without the peptides were subtracted from those with the peptides in solution to eliminate scattering.

3.2.4 LPS binding assay

The ability of the test peptides to bind to lipopolysaccharide (LPS) was determined by assessing their ability to displace bound dansyl polymyxin B (DPX) from LPS (Moore *et al*, 1986). The test solution was made by mixing 2mL of 3μg/mL LPS in 5mM HEPES buffer (pH 7.2), and 20μL of 100μM DPX. The LPS was almost saturated with DPX (>90%). The LPS used was isolated from *E. coli* UB1005 at the Department of Microbiology and Immunology at the University of British Columbia (Moore *et al*, 1986).

The fluorescence was measured using a Perkin Elmer Luminescence Spectrometer (Model LS50B). The excitation and emission wavelengths were 340nm and 485nm respectively. The solution was titrated with small, equal amounts of the test peptide and the fluorescence was recorded after each addition. If the peptides were able to displace some DPX from LPS, then the fluorescence decreased.

From the data collected the inverse of the percentage of the fluorescence inhibited was plotted against the inverse of the peptide concentration, and a straight line was fitted. From the equation of the line it was possible to calculate the maximum percentage of DPX that could be displaced by the peptides I_{max} (I_{50}=-1/x-intercept). Three runs were carried out for each peptide. The mean I_{max} and I_{50} values and the 95% confidence intervals of the means were calculated for each peptide using Microsoft Excel. To determine if there were differences between the means of the different test peptides the Excel Student T-test function was used. A p-value of less than 0.05 was considered significant.

3.2.5 Outer membrane permeabilisation

The ability of the test peptides to permeabilise the outer membrane of *E. coli* UB1005 was assessed by measuring their ability to promote uptake of 1-*N*-phenylnapthylamine (NPN) into intact cells (Loh *et al*, 1984). For these tests, *E. coli* UB1005 cultures grown to an optical density of 0.5 at 600nm were used. The cells were collected by centrifugation (Sorvall SS34, 10,000rpm, 5 minutes) and washed with 5mM HEPES buffer containing 5μM CCCP

(carbonylcyanide *m*-chlorophenyl-hydrazone) and 5mM glucose, then re-suspended in this buffer to the same optical density.

The fluorescence was measured using the luminescence spectrometer (Perkin Elmer Luminescence Spectrometer - Model LS50B), with excitation and emission wavelengths of 350nm and 420nm, respectively. The initial suspension was made by mixing 1mL of cell suspension and 20μL of 0.5mM NPN, and the initial fluorescence was recorded. The suspension was titrated with small, equal amounts of the test peptide, and the fluorescence was recorded. If the test peptides enabled the uptake of NPN into the cells, then the fluorescence increased.

As a control, the NPN uptake caused by the antibiotic polymyxin B at a concentration of 4μg/mL was also determined. The NPN uptake caused by the peptides was expressed as a percentage of the NPN uptake caused by this control, and this percentage of NPN uptake was plotted against the peptide concentration. The assay was carried out three times. The mean NPN uptakes at each concentration and the 95% confidence intervals for the means were calculated using Microsoft Excel and were plotted using SigmaPlot.

3.2.6 Cytoplasmic membrane depolarisation

The ability of the test peptides to interact with the bacterial cytoplasmic membrane was determined by investigating their ability to depolarise this membrane (Wu and Hancock, 1999b). The fluorescence of the membrane potential-sensitive dye 3,3-dipropylthiacarbocyanine ($DiSC_35$) was monitored when added to the *E. coli* DC2, which is an outer membrane permeable mutant. This strain was used to permit the assay to be performed in the absence of an outer membrane permeabilising agent like EDTA. The culture was grown to an optical density of 0.5 at 600nm. The cells were collected using centrifugation (Silencer, 3000rpm, 10 minutes), and washed with 5mM HEPES buffer containing 5mM glucose, then re-suspended in this buffer to the same optical density. To introduce the dye into the cells, 45mL of the cell suspension was incubated with 45μL of 0.4mM $DiSC_35$ (final $DiSC_35$ concentration of 0.4μM) at room temperature with mixing for 30 minutes. To stabilise the inner membrane polarity, 5mL of 1M KCl was added to the suspension and it was incubated for a further 10 minutes at room temperature with mixing.

The initial fluorescence of the suspension containing the cells, $DiSC_35$ and KCl, was measured with the excitation wavelength set to 622nm and the emission wavelength set to

665nm (Perkin Elmer Luminescence Spectrometer - Model LS50B). The suspension was titrated with small, equal amounts of the test peptide, and the fluorescence was recorded. If the test peptides caused the release of $DiSC_35$ from the cells then the fluorescence increased.

As a control, the $DiSC_35$ release caused by the antibiotic gramicidin S at a concentration of 4μg/mL was also determined. The $DiSC_35$ release caused by the peptides was expressed as a percentage of the $DiSC_35$ release caused by this control, and this percentage of $DiSC_35$ release was plotted against the peptide concentration. The assay was carried out three times. The means of the three runs and the 95% confidence intervals for the means were calculated using Microsoft Excel and were plotted using SigmaPlot.

3.2.7 Optical density and viable cell counts over time

To determine how quickly the peptide acted on Gram-negative bacteria the viable cell numbers and the culture optical density were monitored over time. Log phase *E. coli* O111 cells were diluted in MHB to an optical density at 600nm of approximately 0.1. The culture was split between 5 cuvettes, each containing 2mL. The cultures were incubated in a water bath at 37°C. At time zero, the peptides were added so that their final concentrations were twice their MICs (4μg/mL for SMAP29, 4μg/mL for OaBac5mini, 32μg/mL for OaBac7.5mini). As a negative control, one sample contained 10μL of 0.01% acetic acid, and as a positive control, one sample contained 4μg/mL polymyxin B. The optical density at 600nm of each sample was recorded initially and at various times up to two hours after the start of the assay. At each time point 100μL was taken from each sample, and ten-fold serial dilutions up to 10^{-7} were made and plated on Brain-Heart Infusion agar. The plates were incubated overnight at 37°C, and the plates containing the appropriate dilutions were counted the following day.

3.2.8 Peptide-DNA binding

To determine whether the peptides were able to bind to DNA, the ability of the DNA to migrate on an agarose gel was monitored (Park *et al*, 1998). The test solutions were made by mixing 100ng of DNA (Lambda DNA Eco markers) with various amounts of test peptide up to 400ng in 20μL of binding buffer (10mM Tris-HCl (pH8), 20mM KCl, 1mM EDTA, 1mM DTT, 5% glycerol, 50μg/mL BSA). The solutions were left to react for 1 hour. To trace the DNA, 2μL of loading buffer (20% sucrose, 0.125% bromophenol blue) was added to each

sample before they were loaded onto a 0.75% agarose/TAE gel. The gel was run at 108V and 92A for approximately 1 hour. The running buffer used was TAE buffer (0.04M Tris-acetate, 0.001M EDTA). The gel was removed from the apparatus and stained with a drop of ethidium bromide in water for 20 minutes. The gel was placed over a fluorescent light to make the bands visible. The gel was photographed with a digital camera to record the results.

3.3 MATERIALS AND METHODS USED TO INVESTIGATE BACTERIAL CELL MORPHOLOGICAL CHANGES

3.3.1 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the morphology of bacterial cells treated with the test peptides. TEM works in a similar way to regular light microscopy, except that instead of using a focussed beam of light to see through the samples, a focussed beam of electrons is used. Light microscopy is limited, because of the physics of light, to 1000 times magnification and 0.2μm resolution, whereas the electron microscope used for this work was capable of magnifications up to 70,000 times.

Log phase cultures of *E. coli* O111 and *S. aureus* NCTC 4163 in MHB were split into 1.5mL aliquots. The cells were collected by centrifugation (10,000rpm, 5mins) and re-suspended in peptone water. For each organism 6 samples were prepared; two untreated, two treated with SMAP29 (4μg/mL for both organisms) and two treated with OaBac5mini (4μg/mL for *E. coli* O111 and 64μg/mL for *S. aureus* NCTC 4163). The samples were incubated at 37°C for one hour. The cells were collected by centrifugation (10,000rpm, 5mins) to remove the peptone water. To fix the cells, 2% glutaraldehyde in 0.1M cacodylate was added and the samples were incubated at 4°C for one hour. The cells were collected by centrifugation (10,000rpm, 5mins) and washed twice with 0.1M phosphate buffer. To postfix the cells, 1% osmium tetraoxide was added and the samples were left at room temperature for one hour. The samples were dehydrated with graded ethanol solutions (30% ethanol for 10mins, 60% ethanol for 10mins, 90% ethanol for 10mins, 100% ethanol for 10mins, 100% ethanol for 1 hour), embedded in Procure 812 resin and left to polymerise for 48 hours. Thin slices (approximately 100nm) of the samples were made with a diamond knife and stained with uranyl acetate and lead citrate on grids. For each sample ten sections were cut. Each of the sections was examined with a Philips EM201 80kV Transmission Electron Microscope and images were taken with a 35mm camera.

3.3.2 Atomic force microscopy

Atomic force microscopy (AFM) was used to investigate the surface morphology of bacterial cells treated with the test peptides. AFM uses a cantilever with a sharp tip to probe the surface of the sample to gather information about its topology. The cantilever is between 100 and 200μm long and the tip is approximately 2μm long and less than 100Å in diameter. When the tip and the sample come into close proximity, the force between the two causes the cantilever to bend or deflect. As the tip scans over the sample the movement in the cantilever is detected by using a laser beam that bounces off the sample onto a position-sensitive photodetector. This information is used to generate a map of the surface topography of the sample.

Log phase cultures of *E. coli* O111 and *S. aureus* NCTC 4163 in MHB were split into 1mL aliquots. The cells were collected by centrifugation (10,000rpm, 5mins) and resuspended in peptone water. For each organism one sample was untreated, one was treated with SMAP29 (4μg/mL for both organisms) and one was treated with OaBac5mini (4μg/mL for *E. coli* O111 and 64μg/mL for *S. aureus* NCTC 4163). These peptide concentrations were four times the MICs for the given organisms. The samples were incubated at 37°C for 30 minutes. The cells were collected by centrifugation (10,000rpm, 5mins) to remove the peptone water.

A number of different methods were tried to secure the cells for AFM imaging. These included passing the samples through polycarbonate membranes (SPI-Pore Filter, 13mm diameter, 0.8μm pores) to entrap the cells in the pores, and immobilising the cells in agarose gel (5% agarose). The most successful method used involved drying the culture on a glass slide. The treated samples were washed with, and resuspended in, water, and then diluted 100-fold. Approximately 10μL of the sample was dropped onto a glass microscope slide and allowed to dry at room temperature. For each sample duplicate slides were prepared.

To image the samples an Asylum Research MFP-3D scanning probe microscope was used. The tips used were either NT-MDT CSG01 or Olympus TR400PSA, which typically have spring constants in the range 0.01-0.08 N/m. The areas for imaging were chosen randomly from the total sample area.

3.4 MATERIALS AND METHODS USED TO ASSESS THE EFFECT OF CONDITIONS ON PEPTIDE ACTIVITY

3.4.1 Salt effects

The effect of salt concentration on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides against *E coli* O157:H7 in a variety of salt concentrations. The modified micro-broth dilution assay described in Section 3.2.2 was used. The MHB used in the assay was altered by the addition of NaCl. The final NaCl concentrations desired were 0, 50, 100 and 250mM, so MHB solutions containing 0, 100, 200 and 500mM NaCl were required, because the media were diluted with equal amounts of the peptide solution in the assay. Three runs each containing duplicates of each sample were carried out. The geometric means and 95% confidence limits of the means of the MICs were calculated in the way described in Section 3.2.2.

The MIC data from each experiment were analysed to determine if there was a difference between the mean MICs for the different peptides and for the different conditions. The "general analysis of variance" function in statistical programme GenStat was used. The y-variate was "Ln MIC", the treatment structure was "condition x peptide" and the blocks was "run number". From the output, the F-statistics for the condition and peptide were calculated. For these calculations the interaction error term was used instead of the residual error term. This was done because the residual error was likely to be an underestimate of the variability of the data because the data were discrete. Moreover, the use of the interaction error was a more conservative approach. The F-statistics were used to calculate the probability that the means of each data set were the same.

3.4.2 Cation effects

The effect of cation concentration on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides against *E. coli* O157:H7 in a variety of cation concentrations. This was accomplished in the same way as described in Section 3.4.1 for determining the effect of the salt concentration on the activity of the peptides. The two monovalent cations, Na^+ and K^+, and two divalent cations, Ca^{2+} and Mg^{2+}, were added to the media as chloride salts. The concentrations of these cations in the assays were 0, 2, 5 and 10mM. The statistical analysis was carried out using the method described in Section 3.4.1.

To directly compare the means of two sets of data the Student's t-test was used. This was done using the "TTEST" function in Excel for a one-sided, two-sample, equal variance test.

3.4.3 pH effects

The effect of pH on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides against *E. coli* O157:H7 at a variety of pH values. This was achieved by using the micro-broth dilution MIC method described in Section 3.2.2 and altering the pH of the media by the addition of concentrated HCl or NaOH. The peptides were tested in pH conditions that varied from pH5 to pH9. The statistical analysis was carried out using the method described in Section 3.4.1.

3.4.4 Temperature effects

The effect of temperature on the antimicrobial activity of the peptides was tested by determining the MIC of the peptides against *E. coli* O157:H7 after they had been heated to a variety of temperatures. The MICs were determined using the micro-broth dilution MIC method described in Section 3.2.2. Before the MIC assay, the peptides were heated for 30 minutes and then cooled. Temperatures of 30, 40, 50, 60, 70 80 and 90°C were used. The samples were also autoclaved (121°C, 20 minutes). The statistical analysis was carried out using the method described in Section 3.4.1.

3.4.5 Synergistic effects between test peptides

To test whether the peptides worked synergistically against *E.* coli O157:H7, checkerboard titrations were used (Fidai *et al*, 1997). One compound was diluted down the columns and the other was diluted across the rows in a 96-well polypropylene microtitre plate. Each well contained 25μL of one peptide diluted in a solution containing 0.2% bovine serum albumin (BSA) and 0.01% acetic acid, and 25μL of the other peptide diluted in the same solution. To each well, 50μL Mueller-Hinton broth (Difco) containing $2x10^5$ cells was added, to give a final concentration of $1x10^5$ cells. The plates were incubated overnight at 37°C.

The next day the wells in which bacterial growth occurred were recorded. The fractional inhibitory concentrations (FICs) were calculated using the formula: $FIC = [A]/MIC_A + [B]/MIC_B$. MIC_A and MIC_B were the MICs of peptides A and B alone. These were the lowest concentration of each peptide that could inhibit the growth of the culture when the

concentration of the other peptide was zero. [A] and [B] were the MICs of peptides A and B when in combination. Each pair of [A] and [B] values from the same well were used to calculate the FIC. The FIC reported is the lowest FIC value calculated from the plate. Three independent runs were carried out. The FICs were the same for each run so no statistical analysis was carried out.

3.4.6 Synergistic effects between test peptides and common antibiotics

The checkerboard titration method described in Section 3.4.5 was used to test whether the test peptides worked synergistically with common antibiotics against *E. coli* O157. The common antibiotics tested in combination with the test peptides were polymyxin B, ampicillin, kanamycin A and rifampicin. These antibiotics were chosen because they each have different mechanisms of action. The FICs of each antibiotic with each test peptide were determined three times in separate runs. The FICs were the same for each run so no statistical analysis was carried out.

3.5 MATERIALS AND METHODS USED FOR THE PILOT-SCALE EXTRACTION OF ANTIMICROBIAL PEPTIDES FROM OVINE BLOOD

3.5.1 Crude extraction process

A number of pilot-scale extraction runs were carried out to determine whether the crude extract could be produced on a larger-scale than that used in the laboratory. For these extractions 10-50L of blood was used compared to 2-4L in the laboratory extractions. The pilot-scale extraction of antimicrobial peptides used a modified version of the laboratory extraction described in 3.1.1. Full details of this entire process are given in Chapter 8.

The first step in the extraction process was to separate the white blood cells from the rest of the blood. For the pilot-scale extractions the intention was to use the modified lab-scale process based on that of the University of California, Los Angles group (Borenstein *et al*, 1991) to lyse the red blood cells. However, during experimentation it was discovered that it was possible to separate most of the red blood cells from the white blood cells using centrifugation only. A continuous-feed disk-stack centrifuge (Alfa-Laval Cream Separator), usually used for milk separation, was used. When the whole blood was passed through the centrifuge the plasma came out the liquid stream, most of the red blood cells came out the concentrated solids stream, and the white blood cells and the rest of the red blood cells were

pelleted inside the centrifuge. The white blood cells and residual red blood cells were suspended in PBSX buffer (137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.4). The contaminating red blood cells were lysed using the ammonium chloride method, and then the white blood cells were collected using centrifugation.

Once the white blood cells were separated from the rest of the blood the next step in the extraction process was to isolate the granules, which contained the antimicrobial peptides, from the neutrophils. The original lab-scale process used ultrasonic energy to disrupt the white blood cells and release the neutrophil granules (Eisenhauer *et al*, 1989). The granules were then collected using high-speed centrifugation and dissolved in acetic acid solution. However, due to the large amount of heat generated by sonication, this operation would be impractical for a large-scale process. Therefore, an alternative method to disrupt the white blood cells was required. Mechanical disruption using a bench-top blender was tested and it was found to be as effective as the sonication method. The yield from an extraction using the sonicator was the same as the yield from an extraction using the blender.

A further modification was introduced to the pilot-scale extraction process. Instead of resuspending the pelleted white blood cells in PBSX buffer, acetic acid solution was used. This eliminated the need for the centrifugation step to pellet the granules so they could be resuspended in the acetic acid solution. This meant that the white blood cell debris was not removed prior to the acid extraction step; however, this did not affect the process.

The next step in the process was to extract the antimicrobial peptides from the neutrophil granules. In both the lab-scale and pilot-scale processes the granules were suspended in 10% acetic acid and left overnight at 4°C with constant mixing. However, these conditions were not determined experimentally, so further work is required to optimise the extraction. Both the extraction solution and the extraction method should be investigated to determine the most effective method for extracting the antimicrobial peptides.

The final step in the process was to convert the product of the acid extraction into a crude antimicrobial extract that was stable for use and storage. This involved the removal of the neutrophil granules and other suspended solids, removal of the acetic acid and concentration of the solution. In both the lab-scale and pilot-scale processes the formulation of the crude extract was carried out in the same way. After centrifugation to remove the solids rotary evaporation was used to remove the acid and freeze-drying was used to remove the water.

Then the product was dissolved to form the crude extract. These acid and water removal steps were used because they did not require heating, which may have damaged the product.

3.5.2 Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of the crude extract against a variety of organisms were determined using the modified micro-broth dilution method described in Section 3.2.2. The test organisms included eight Gram-negative bacteria, eight Gram-positive bacteria and one yeast. The test organisms and there sources are listed in Table 3.3

Table 3.3 - Microorganisms used for crude extract minimum inhibitory concentration tests and their sources

Microorganism	Source
Escherichia coli O111	Communicable Disease Centre, New Zealand.
Escherichia coli O157:H7	Communicable Disease Centre, New Zealand.
Salmonella enteritidis	Institute of Veterinary, Animal and Biomedical Sciences, Massey University, New Zealand.
Salmonella typhimurium	Institute of Technology and Engineering, Massey University, New Zealand.
Klebsiella pneumoniae	Institute of Technology and Engineering, Massey University, New Zealand.
Pseudomonas aeruginosa NCTC 6749	National Collection of Type Cultures, England.
Pseudomonas fluorescens ATCC 13525	American Type Culture Collection, USA.
Yersinia enterocolitica ATCC 9610	American Type Culture Collection, USA.
Staphylococcus aureus NCTC 4163	National Collection of Type Cultures, England.
Staphylococcus aureus 1056 MRSA	Institute of Food, Nutrition and Human Health, Massey University, New Zealand.
Staphylococcus faecalis NHI 89	National Health Institute, New Zealand.
Bacillus cereus NCIB 8709	National Collection of Industrial Bacteria, Scotland.
Bacillus nato	Institute of Food, Nutrition and Human Health, Massey University, New Zealand.
Listeria monocytogenes 108 A	Institute of Food, Nutrition and Human Health, Massey University, New Zealand.
Listeria monocytogenes NCTC 10884	National Collection of Type Cultures, England.
Listeria monocytogenes NCTC 7973	National Collection of Type Cultures, England.
Candida albicans 3153A	Institute of Molecular Biosciences, Massey University, New Zealand.

Two-fold serial dilutions of the extract at concentrations between 128 and 0.0625 μL crude extract/mL were tested. The MIC was defined as the concentration of the peptide in the last well in which culture growth did not occur. The MIC values could not be expressed in μg/mL because the mass of the solids in the crude extract was not comparable to the activity due to the presence of other non-active proteins and peptides in unknown concentrations. Instead, the MICs were described in units/mL, where one unit was defined as the amount required to inhibit 10^5 CFU/mL *E. coli* O111. The assay was carried out three times independently. For each run duplicates of each sample were included. The statistical analysis was done as described in Section 3.2.2.

3.5.3 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the morphology of bacterial and yeast cells treated with the test peptides. Log phase cultures of *E. coli* O111, *S. aureus* NCTC 4163 and *C. albicans* 3153A in MHB are split into 1mL aliquots. The cells were collected by centrifugation (10,000rpm, 5mins) and resuspended in peptone water. For each organism there were four samples prepared; two untreated (0.01% acetic acid) and two treated with 10μL of crude extract. The samples were incubated at 37°C for one hour. The samples were prepared for TEM and photographed as described in Section 3.3.1.

3.5.4 Yield calculations

The yield of units of activity was calculated using Equation 3.2 for each extraction. This is the number of units of activity produced from each litre of blood, where one unit is the amount required to inhibit 10^5 CFU/mL *E. coli* O111.

$$\text{yield} = \frac{\text{volume of extract} / \text{volume per unit}}{\text{volume of blood}} \qquad \textbf{Equation 3.2}$$

where:

volume of extract = the volume of crude extract produced from the run (mL)

volume per unit = the volume of crude extract required for one unit of activity (mL)

volume of blood = the volume of blood processed for the run (L)

CHAPTER 4
ISOLATION AND CHARACTERISATION OF ANTIMICROBIAL PEPTIDES FROM OVINE NEUTROPHILS

4.1 INTRODUCTION

The work presented in this chapter was concerned with the first objective of this research project, which was to purify and identify antimicrobial peptides from ovine blood. Prior work showed that it was possible to extract components from ovine neutrophil granules that have broad-spectrum antimicrobial activity (Anderson and Yu, unpublished results). The first step of the current research was to separate and identify the components in the crude extract to determine the components responsible for the antimicrobial activity.

It was hypothesised that most of the antimicrobial activity would be caused by cathelicidin peptides. As discussed in the literature review in Chapter 2, cathelicidins are a family of gene-encoded cationic antimicrobial peptides found only in mammals. These peptides are stored as inactive propeptides in neutrophil granules and are cleaved into active peptides by neutrophil elastase when required. Cathelicidins all share a strongly conserved N-terminal precursor, similar to that of porcine cathelin (hence the name). However, the C-terminal regions of these molecules, which are bactericidal, are highly variable.

Using this knowledge of the cathelin-like domain, eight ovine genes encoding seven different cathelicidins have been identified: two α-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). Until now, only two variants of the predicted peptide OaBac5, have been isolated from ovine neutrophils (Shamova *et al*, 1999). Another two of the predicted peptides, SMAP29 and SMAP34, have been synthesised by several groups, and one of these, SMAP29, has potent, broad-spectrum antimicrobial activity (Skerlavaj *et al*, 1999; Travis *et al*, 2000).

The objective of the research presented in this chapter was to isolate the components in the ovine neutrophil crude extract that displayed antimicrobial activity in the earlier study (Anderson and Yu, unpublished results, 2000). Once isolated, the molecular weights of the compounds were determined by mass spectroscopy, and their N-terminal sequences were determined and compared with those of known peptides and proteins.

4.2 EXTRACTION OF CRUDE ANTIMICROBIAL SOLUTION

The process outlined in Figure 4.1 was used to obtain the crude antimicrobial extract from the ovine blood. This process was developed by combining ideas from numerous processes presented in the literature as described in Section 3.1.1. Over the course of this research the extraction process was modified several times to make it quicker, easier and more economical.

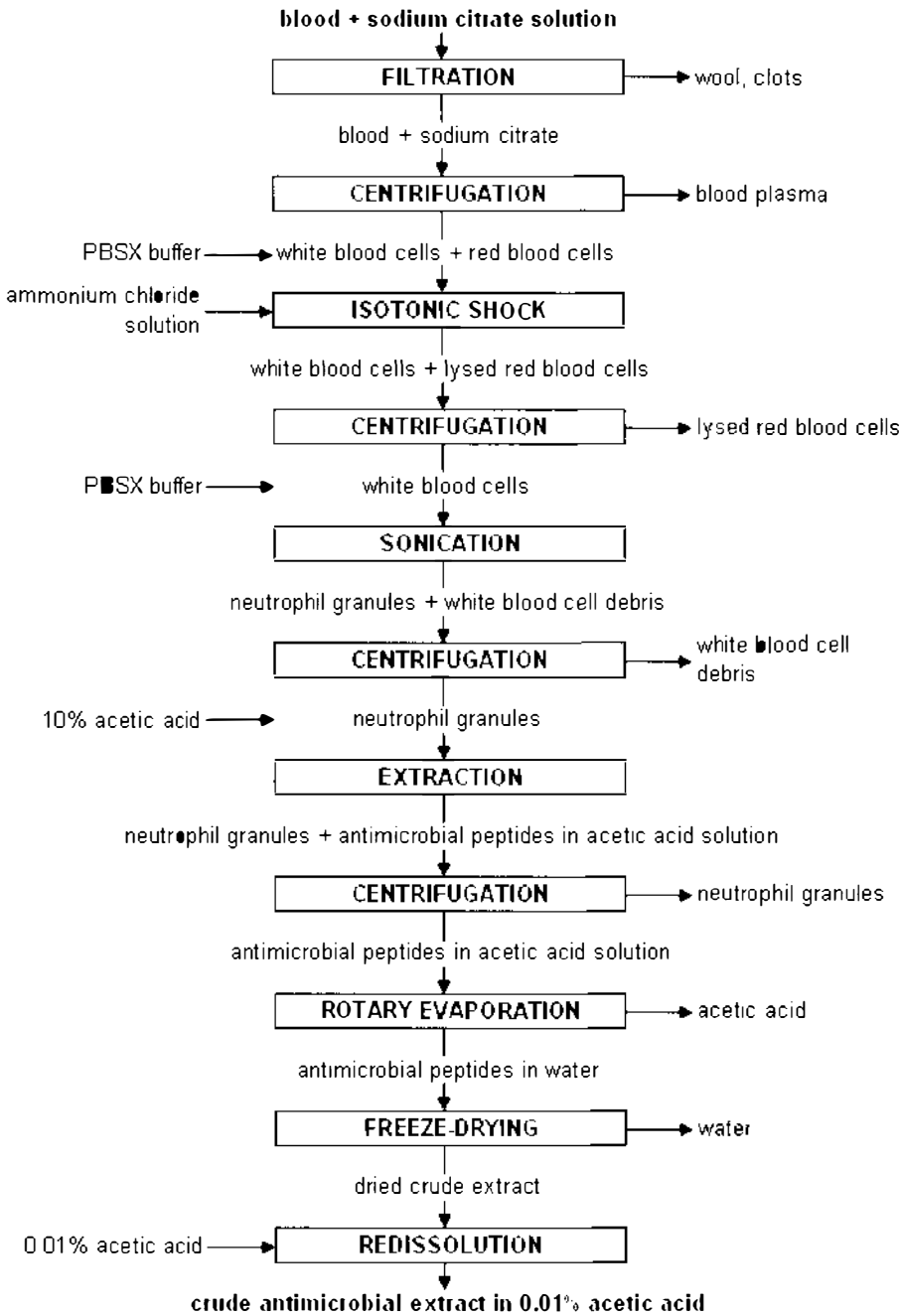

Figure 4.1 - Flowchart showing the process used to extract the crude antimicrobial solution from ovine blood. Sodium citrate solution contained 10% sodium citrate. PBSX buffer contained 137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 (pH 7.4). Ammonium chloride solution contained 0.83% ammonium chloride.

Throughout the course of this research the extraction process was carried out approximately fifteen times, with between two and four litres of ovine blood being processed each time. To ensure the steps in the process worked effectively, samples were taken after each critical step and examined. The blood cells were stained using a Quick-Diff staining kit then viewed under a microscope. Images of the blood at various stages during the extraction process are given in Figure 4.2. The whole blood contained numerous small red blood cells and some larger white blood cells. If red blood cells were still present in the blood after the lysis step, this step was repeated as outlined in the methodology in Section 3.1.1. The white blood cell disruption step was similarly repeated if white cells were still present after the disruption step.

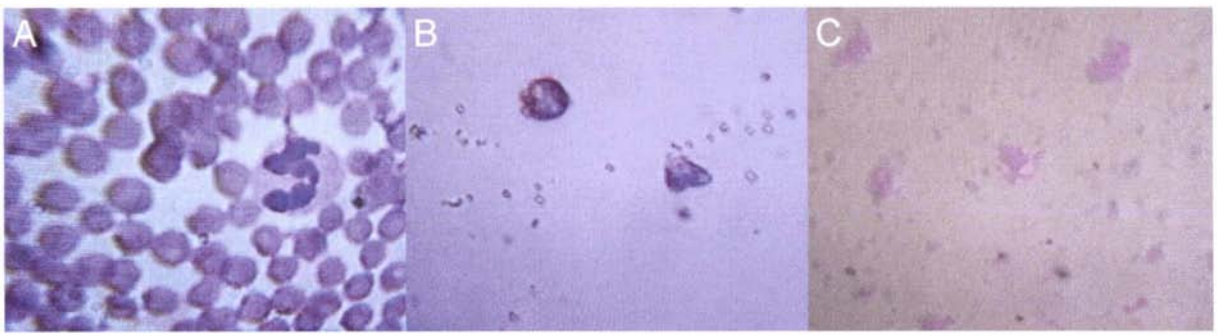

Figure 4.2 - Images of typical stained blood samples during the extraction process. 'A' shows whole ovine blood, 'B' shows ovine blood after the red blood cell lysis step, and 'C' shows ovine blood after the white blood cell disruption step.

After each extraction, the antimicrobial activity of the crude extract was tested to determine whether the extraction was successful. To do this the radial diffusion plate assay method was used. This method is described in Section 3.1.6. The test organisms used were *E. coli* O111, *S. aureus* NCTC 4163 and *C. albicans* 3153A. These organisms were chosen to indicate the spectrum of activity of the extract because they are a Gram-negative bacterium, Gram-positive bacterium and yeast respectively. Photographs of a typical set of plate assays for the crude extract are shown in Figure 4.3.

To separate the components in the crude extract, the extract was fractionated using either gel filtration or cationic exchange chromatography. The active fractions were determined using the radial diffusion plate assay method, and were further processed using RP-HPLC to separate the individual components. These RP-HPLC fractions were then subjected to a radial diffusion plate assay to determine which ones were active, and these pure active components were then analysed. The characterisation of antimicrobial peptides via the gel

filtration purification method will be discussed first, followed by the characterisation via the cationic exchange method.

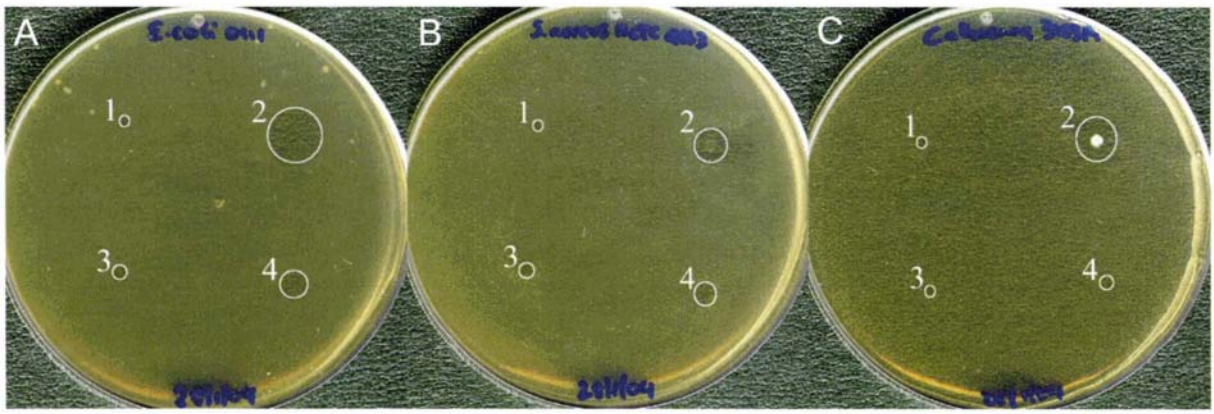

Figure 4.3 - Images of typical plate assay results of neutrophil crude extract against three test organisms. 'A' shows *E. coli* O111, 'B' shows *S. aureus* NCTC 4163 and 'C' shows *C. albicans* 3153A. Well '1' contains the negative control (0.01% acetic acid), '2' contains the positive control (1μg/mL polymyxin for *E. coli* O111, 1μg/mL nisin for *S. aureus* NCTC 4163, and 10μg/mL nystatin for *C. albicans* 3153), '3' contains ovine neutrophil crude extract diluted 1/10, and '4' ovine neutrophil extract undiluted. The white circles are computer graphics to highlight the clearings.

4.3 PURIFICATION OF ANTIMICROBIAL PEPTIDES USING GEL FILTRATION AND RP-HPLC

The crude extract was passed through a P10 gel filtration column to separate the components in the solution according to their sizes using the method described in Section 3.1.3. A typical chromatograph is shown in Figure 4.4. This P10 resin, which has a molecular weight cut-off of 10kDa, was chosen because it was thought that the active components were cathelicidin peptides, the majority of which have a molecular weight between 3 and 5kDa. The running buffer contained 5% acetic acid and no salt. Salt was not added to the buffer because it was too difficult to remove the salt from the solutions after the fractions were concentrated without losing the active components. It was undesirable to keep the salt in the solutions because it reduces the antimicrobial activity of the peptides as demonstrated in Section 7.2.

The plate assay method was used to determine which gel filtration fractions had antimicrobial activity. Before testing, each fraction was rotary evaporated to remove the majority of the acid, freeze-dried to remove the residual acid and water, and then dissolved in 2-5mL 0.01% acetic acid. The results of a plate assay of the gel filtration fractions from a typical run are given in Table 4.1. The earliest fractions to elute from the gel filtration column (F1, F2 and F3) had antimicrobial activity against the three test organisms, *E. coli* O157:H7, *S. aureus*

1056 MRSA and *C. albicans* 3153A, but the fractions that eluted later (F4, F5 and F6) were not active against any of the test organisms. Fraction 2 was the most active fraction, followed by Fraction 1 and then Fraction 3.

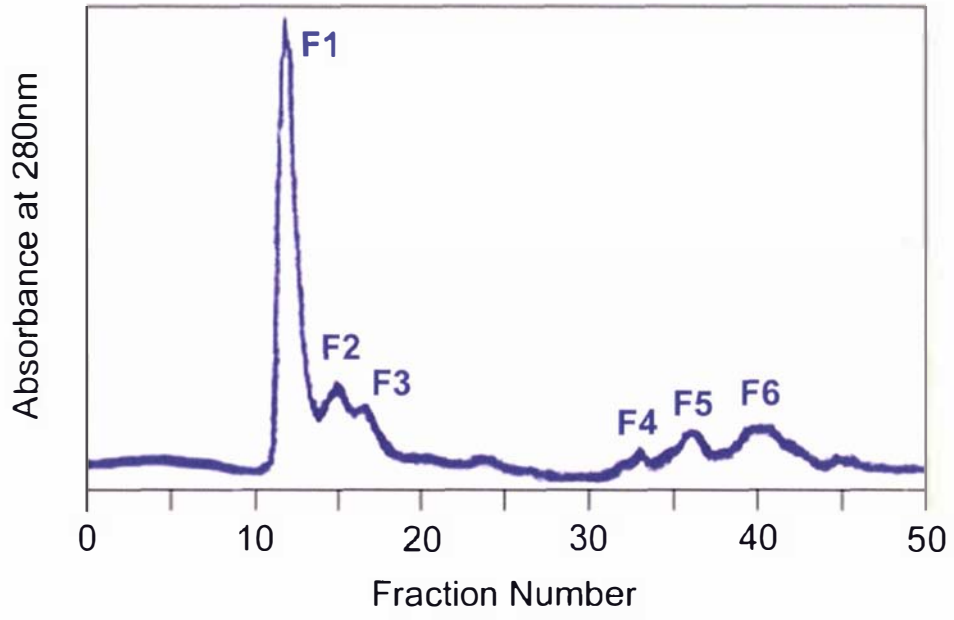

Figure 4.4 - Typical gel filtration chromatograph resulting from the addition of an ovine neutrophil crude extract into a P10 gel filtration column. The running buffer was 5% acetic acid, which was pumped through the column at a rate of 20mL/hour. Each fraction contained 5mL of liquid.

Table 4.1 - Antimicrobial activity of typical ovine neutrophil extract gel filtration fractions against test organisms.

Test solution	Diameters of clearings on plate assays (mm)		
	E. coli O157:H7	*S. aureus* 1056 MRSA	*C. albicans* 3153A
positive control*	18	9	13
F1	8	7	6
F2	12	8	7
F3	6	4	2
F4	no clearing	no clearing	no clearing
F5	no clearing	no clearing	no clearing
F6	no clearing	no clearing	no clearing
negative control**	no clearing	no clearing	no clearing

*** The positive controls were 1μg/mL polymyxin for *E. coli* O157:H7, 1μg/mL nisin for *S. aureus* 1056 MRSA, and 10μg/mL nystatin for *C. albicans* 3153A.**
**** The negative control was 0.01% acetic acid.**

SDS-PAGE was used to determine the number of components in each gel filtration fraction and their approximate molecular weights according to the method described in Section 3.1.2. An image of a typical SDS-PAGE gel is given in Figure 4.5. This showed that F2, the most active fraction, contained molecules of the expected size for cathelicidin peptides (3-5kDa). Interestingly, F3 and F4 contained components that were larger than those in F2, even though these fractions eluted from the gel filtration column after F2. The P10 resin was supposed to separate components smaller than 10kDa, and larger molecules, such as those present in F3 and F4, should have passed straight through the column. The large molecular weights of the molecules in F3 and F4 were probably not due to multimers forming during electrophoresis because the β-mercaptoethanol should have reduced the bonds. These large components may have had reduced migration speeds during gel filtration as a consequence of their interactions with the resin.

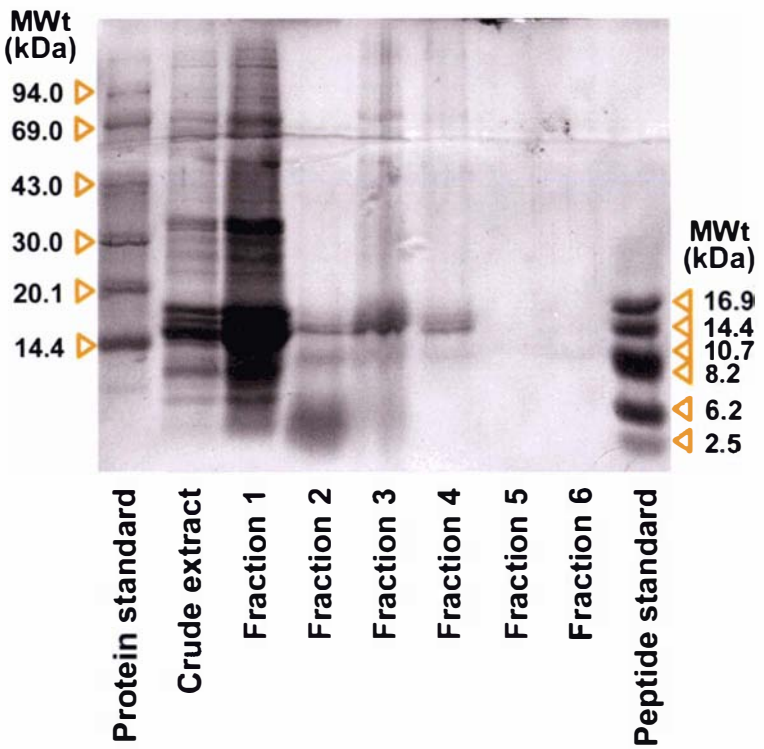

Figure 4.5 - Image of a typical SDS-PAGE gel of ovine neutrophil extract gel filtration fractions.

The second gel filtration fraction (F2) was chosen for further purification because it had the strongest antimicrobial activity and because it contained molecules of the expected size. F1 also had good antimicrobial activity and it contained some low molecular weight compounds; however, it contained numerous large proteins that made further separation difficult. It was assumed that the low molecular weight compounds present in F1 were the same as those in F2 because the separation of the fractions was not clean.

The components in the second gel filtration fraction (F2) were separated using RP-HPLC as described in Section 3.1.5. Initially a gradient of 5-95% acetonitrile was run and the peaks were collected in groups. Plate assays were used to determine the active region, and trial and error was used to determine the best acetonitrile gradient to separate these components. The resulting chromatograph is shown in Figure 4.6. Each of the peaks was collected manually, freeze-dried and redissolved in 30µL of 0.01% acetic acid.

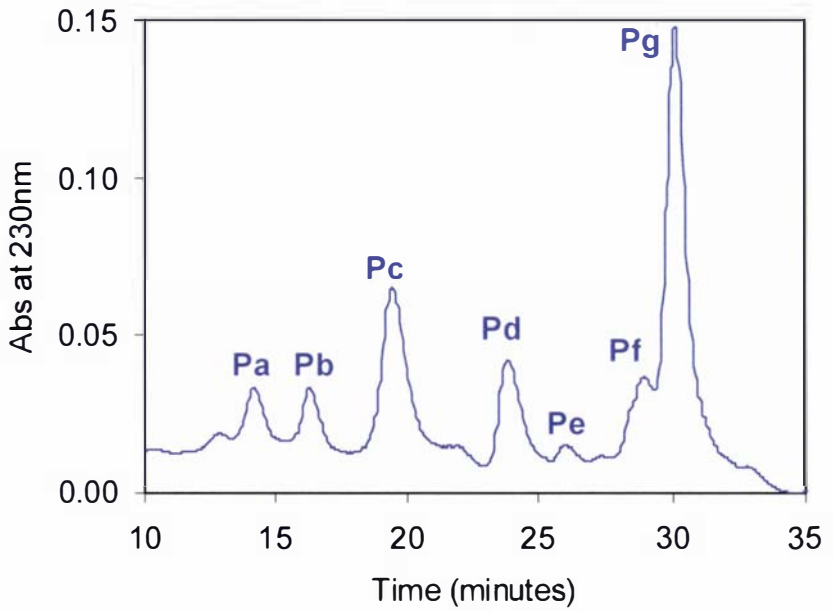

Figure 4.6 - RP-HPLC chromatograph of the second gel filtration fraction (F2) of the ovine neutrophil crude extract. Buffer A contained 5% acetonitrile and 0.1% trifluroacetic acid (TFA). Buffer B contained 95% acetonitrile and 0.1% TFA. A gradient of 28% to 32% of Buffer B over 30 minutes was used.

Table 4.2 - Antimicrobial activity of RP-HPLC peaks from the second gel filtration fraction of the ovine neutrophil crude extract against test organisms.

Test Solution	Diameter of clearings on plate assays (mm)		
	E. coli O157:H7	*S. aureus* 1056 MRSA	*C. albicans* 3153A
positive control*	14	7	10
peak a	6	4	3
peak b	5	3	4
peak c	7	4	4
peak d	4	3	5
peak e	no clearing	no clearing	no clearing
peak f	3	no clearing	no clearing
peak g	no clearing	no clearing	no clearing
negative control**	no clearing	no clearing	no clearing

* **The positive controls were 1µg/mL polymyxin for *E. coli* O157:H7, 1µg/mL nisin for *S. aureus* 1056 MRSA, and 10µg/mL nystatin for *C. albicans* 3153A.**

** **The negative control was 0.01% acetic acid.**

The plate assay method was used to establish whether each of the samples that corresponded to a peak on the HPLC chromatograph was able to inhibit the growth of the test organisms. The results are given in Table 4.2. Five of the seven fractions displayed antimicrobial activity in the plate assays.

The active peaks were characterised using the mass spectroscopy and N-terminal sequencing methods described in Sections 3.1.8 and 3.1.9. The results showed that all of the samples were proline/arginine-rich peptides. Pa and Pc were OaBac5 and a variant of OaBac5 respectively, Pb was a truncated form of OaBac7.5mini, and Pd and Pf were mixtures of truncates of OaBac11. The characterisation of each of these peptides is discussed in detail in the following sections.

4.4 CHARACTERISATION OF OABAC5 AND VARIANTS

Of the five purified components that had antimicrobial activity, two (Pa and Pc) were variants of OaBac5. The molecular weights and N-terminal sequences of Pa and Pc are compared to OaBac5 in Table 4.3. For comparative purposes, this table also gives the sequences of the Bac5 peptides isolated from bovine and goat blood, as well as two variants of OaBac5 that were previously purified from ovine blood.

OaBac5 is a proline/arginine-rich cathelicidin peptide predicted from sheep cDNA (Mahoney *et al*, 1995; Huttner *et al*, 1998). The original sequence was predicted to contain three more residues than those shown (GRR); however, it was assumed that the mature peptide would not contain these residues because glycine is usually an amidation signal in cathelicidins.

As shown in Table 4.3, the N-terminal sequence and mass of the peptide in fraction Pa matched those of the amidated version of OaBac5. The fifth residue was unable to be determined. However, this residue was probably a proline because multiple proline residues are often difficult to sequence (Protein Analysis Facility, Personal Communication, 2002)

The N-terminal sequence of Pc differed from that of OaBac5 by one amino acid at residue 7. The molecular mass of Pc matched the mass predicted for amidated OaBac5 with this change at residue 7 as shown in Table 4.3; therefore, this was assumed to be the complete sequence. This new peptide was named OaBac5γ, because its sequence was different from that of OaBac5α and OaBac5β (Shamova *et al*, 1999).

Table 4.3 - Comparison of masses and N-terminal sequences of Pa and Pc purified from the ovine neutrophil crude extract to known Bac5 peptides.

Peptide	Source	MWt (kDa)	Sequence	Reference
OaBac5	Predicted from ovine cDNA	5170.29[a]	RFRPPIRRPPIRPPFRPPFRPPVRPPIRPPFRPPF RPPIGPFP	Huttner *et al*, 1998
ChBac5	Purified from caprine blood	5162.23	RFRPPIRRPPIRPPFNPPFRPPVRPPFRPPFRPPF RPPIGPFP	Brogden *et al*, 1998
Bac5	Purified from bovine blood	5148.28[b]	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPF RPPLGPFP	Zanetti *et al*, 1993
Pa N-terminal	Purified from gel filtration fraction 2	5169.75[b]	RFRP?IRRPPIRPPFRPPFRPPV.....	This work
Pa proposed/ OaBac5	Proposed based on N-terminal sequence and mass	5170.29[a]	RFRPPIRRPPIRPPFRPPFRPPVRPPIRPPFRPPF RPPIGPFP	
Pc N-terminal	Purified from gel filtration fraction 2	5125.53[b]	RFRP?ILRPPIRPPFRPPFR.....	This work
Pc proposed/ OaBac5γ	Proposed based on N-terminal sequence and mass	5127.27[a]	RFRPPILRPPIRPPFRPPFRPPVRPPIRPPFRPPF RPPIGPFP	
OaBac5α N-terminal	Purified from ovine blood	5157.50[b]	RFRP?IRRPPIRPPFRPPFR.....	Shamova *et al*, 1999
OaBac5α proposed	Proposed based on N-terminal sequence and mass	5156.27[a]	RFRPPIRRPPIRPPFRPPFRPPVRPPVRPPFRPPF RPPIGPFP	
OaBac5β N-terminal	Purified from ovine blood	5119.50[b]	RFRP?ILRPPIIPPFRPPFR.....	Shamova *et al*, 1999
OaBac5β proposed	Proposed based on N-terminal sequence and mass	5118.25[a]	RFRPPILRPPIIPPFRPPFRPPVRPPFRPPFRPPF RPPIGPFP	

[a] The molecular weights were calculated. [b] The molecular weights were obtained experimentally (see Appendix A1 for mass spectra).
The residues that differ from those in the OaBac5 sequence are indicated with underlining

The N-terminal sequences of the previously purified OaBac5α and OaBac5β (Shamova *et al*, 1999) are also included for comparison. The N-terminal sequence of OaBac5α matched that of OaBac5, but the masses were different. This indicated that OaBac5α differed from OaBac5 later in the sequence. If the isoleucine at residue 27 were replaced with a valine, then the peptide mass would match that of OaBac5α; therefore this sequence was proposed. There is another isoleucine further in the sequence that could have been altered instead; however, it is probable that the change occurred at residue 27 because this residue was also altered in the goat homologue (ChBac5) (Shamova *et al*, 1999).

Like Pc/OaBac5γ, the N-terminal sequence of OaBac5β also differed from OaBac5 at residue 7. Residue 12 was also different. The predicted mass of the full peptide that contained these two changes was calculated to be 5084.24Da. However; this was only different to the mass of OaBac5β. Therefore, OaBac5β must have also differed from OaBac5 further in the sequence. If residue 27 was changed from an isoleucine to a phenylalanine, then the peptide mass would match that of OaBac5β. This substitution is likely because it occurs in the goat version of the peptide (Brogden *et al*, 1998).

Four different OaBac5 sequences have now been hypothesised. The suggested sequence differences probably come from genetic reshuffling. One possibility is that a single animal contains multiple copies of the OaBac5 cathelicidin gene, each with a slight variation. However, the examination of ovine cDNA did not uncover more than one OaBac5 gene (Huttner *et al*, 1998). Alternatively, because the blood that was used was pooled from numerous animals, it is possible that the different variations of OaBac5 came from different animals, i.e., each animal may have contained only one copy of the gene, but this gene varied slightly between animals.

4.5 CHARACTERISATION OF TRUNCATED OABAC7.5

One of the peptides purified from the second gel filtration fraction, Pb, had an N-terminal sequence that matched part of OaBac7.5. Table 4.4 shows a comparison between Pb and OaBac7.5. OaBac7.5 is a 60 amino acid proline/arginine-rich peptide predicted from sheep cDNA (Mahoney *et al*, 1995), which has not previously been purified from ovine cells. The N-terminal sequence of Pb matched residues 32 to 51 of OaBac7.5. The complete Pb sequence was probably residues 32 to 60 of OaBac7.5 because the calculated mass was the same as that determined for Pb.

Like OaBac5, OaBac7.5 was also purified in a different form to that predicted. However, the changes were not due to amino acid substitutions, but instead to peptide cleavage. Cathelicidins are stored as propeptides in neutrophils and processed when needed by neutrophil elastase (Zanetti *et al*, 1995). When predicting OaBac7.5 from ovine cDNA, it was assumed that the preproregion would be cleaved from the active peptide, after the valine residue, by neutrophil elastase. However, the peptide purified from ovine neutrophils is much smaller than the predicted OaBac7.5.

This shortened version of OaBac7.5, OaBac7.5$_{(32\text{-}60)}$, may have came about due to cleavage of the complete OaBac7.5 by an enzyme present in the crude extract. Carboxypeptidase E (M14.005 in the MEROPS database) is the only known peptidase that cleaves between a proline and an arginine residue, without other surrounding amino acid specifications that do not fit this peptide. This was determined using the peptidase database as described in Section 3.1.10 This protease is found in the insulin-secreting granules and is involved in the biosynthesis of many peptide hormones and neurotransmitters (Fricker, 1988). If this peptidase were responsible for the cleavage, then it is likely that more cleavages would have occurred, because the Bac peptide sequences contain numerous carboxypeptidase E cleavage sites. For this reason it was thought that the cleavage of OaBac7.5 was carried out by another peptidase, as yet undiscovered, that has a more specific cleavage site.

Interestingly, OaBac7.5 was cleaved where the resulting OaBac7.5$_{(32\text{-}60)}$ peptide began with two arginine residues. This double arginine N-terminus is present in a number of the proline/arginine-rich peptides as discussed in Section 4.8, and it has been shown that the highly cationic N-terminus of Bac7 is crucial for the antimicrobial activity of the peptide (Sadler *et al*, 2002). Therefore the cleavage of OaBac7.5 may not have been accidental.

OaBac11, another ovine proline/arginine-rich cathelicidin, also contains two consecutive arginine residues, which are situated in the fourth repeat. This makes this a potential cleavage site for the peptidase that cleaved OaBac7.5. However, this fragment was not purified from the crude extract.

Table 4.4 - Comparison of mass and N-terminal sequence of Pb purified from the ovine neutrophil crude extract to OaBac7.5.

Peptide	Source	MWt(kDa)	Sequence	Reference
OaBac7.5	predicted from ovine cDNA	7464.10[a]	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPQP RRIPRPILLPWRPPRPIPRPQPQPIPRWL	Huttner *et al*, 1998
Pb N-terminal	purified from gel filtration fraction 2	3600.09[b]	RRIPRPILLPWRPPRPIPRP.....	This work
Pb proposed/ $OaBac7.5_{(32-60)}$	proposed based on N-terminal sequence and mass	3600.42[a]	RRIPRPILLPWRPPRPIPRPQPQPIPRWL	

[a] The molecular weights were calculated. [b] The molecular weights were obtained experimentally (See Appendix A1.1 for mass spectra).

Table 4.5 - Comparison of masses and N-terminal sequences of Pd and Pf purified from the ovine neutrophil crude extract to Bac11.

Peptide	Source	MWt(kDa)	Sequence	Reference
OaBac11	predicted from ovine cDNA	11193.8[a]	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRP LPLPRPRPKPIPRPLPLPRPRPRRIPRPLPLPRPRPR PIPRPLPLPQPQPSPIPRPL	Huttner *et al*, 1998
Pd minor peak/ Pf minor peak	purified from gel filtration fraction 2	11191.4[a] 11191.6[b]	RRLRPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRP LPLPRPRPKPIPRPLPLPRPRPRRIPRPLPLPRPRPR PIPRPLPLPQPQPSPIPRPL	This work
$OaBac11_{(1-46)}$/ Pd minor peak	purified from gel filtration fraction 2	5586.9[a] 5595.5[b]	RRLVPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRP LPLPRPRPK	This work
$OaBac11_{(1-42)}$/ Pd major peak	purified from gel filtration fraction 2	5118.3[a] 5117.1[b]	RRLVPRRPRLPRPRPRPRPRPRSLPLPRPKPRPIPRP LPLPR	This work
$OaBac11_{(1-28)}$/ Pd minor peak	purified from gel filtration fraction 2	3502.3[a] 3503.4[b]	RRLVPRRPRLPRPRPRPRPRPRSLPLPR	This work
$OaBac11_{(1-25)}$/ Pf major peak	purified from gel filtration fraction 2	3192.9[a] 3192.3[b]	RRLRPRRPRLPRPRPRPRPRPRSLP	This work
$OaBac11_{(1-29)}$/ Pf major peak	purified from gel filtration fraction 2	3656.5[a] 3655.5[b]	RRLRPRRPRLPRPRPRPRPRPRSLPLPRP	This work

[a] The molecular weights were calculated. [b] The molecular weights were obtained experimentally (see Appendix A1.1 for mass spectra)

4.6 CHARACTERISATION OF OABAC11 AND TRUNCATES

The final two active HPLC peaks (Pd and Pf) did not have clean mass spectroscopy results because they were a mixture of more than one peptide. Pd contained one major component and three minor components. Pf contained two major and one minor component. The mass spectra are given in Appendix A1. The components of both samples are compared to OaBac11 in Table 4.5.

The N-terminal sequence of Pd was the same as that of OaBac11 and OaBac7.5, except that the 4th residue was predominantly valine instead of arginine. One of the minor components in the mass spectra had a mass similar to the complete OaBac11 as shown in Table 4.5, which suggested that the other peptides may be truncated forms of OaBac11. The mass of the major component in Pd is similar to the mass calculated for the first 42 residues of OaBac11, with the change at the fourth residue (valine instead of arginine) as suggested by the N-terminal sequencing. Similarly, the masses of the other two minor components matched the masses calculated for two other truncates: the first 28 and the first 46 residues of OaBac11, with the change at residue 4.

Like Pd, the mass spectroscopy results of Pf contained a minor peak with a mass similar to OaBac11. N-terminal sequencing was not carried out for this sample because the mass spectra showed that there were two major components instead of one. However, the first 25 and the first 29 residues of OaBac11 (without the change at residue 4) had calculated masses similar to the masses determined for this sample. Therefore, it is likely that Pf was comprised of these two truncated forms of OaBac11.

Like OaBac7.5, OaBac11 was also purified in truncated forms. However, the purified OaBac7.5 truncate was the C-terminus of the complete peptide; whereas, the OaBac11 truncation occurred at the N-terminus of the mature OaBac11 peptide. The peptidase database was searched, as described in Section 3.1.10, to find a peptidase that could cleave at this point. The truncated peptides found in Pd were all cleaved before a proline residue. This cleavage could have been carried out by aminopeptidase P2 (M24.005 in the MEROPS database), which is a eukaryotic membrane-bound peptidase that cleaves prior to a proline residue. In contrast, the truncated peptides found in Pe were all cleaved after a proline residue. OaBac11$_{(1\text{-}29)}$ was cleaved between a proline and arginine residue, so this could have been carried out by carboxypeptidase E, as mentioned for OaBac7.5. However, OaBac11$_{(1\text{-}25)}$

was cleaved between a proline and leucine residue so a different peptidase must have been involved. Lysosomal Pro-X carboxypeptidase (S28.001 in the MEROPS database) could have carried out this cleavage. Alternatively, both truncated forms may have been processed by dipeptidyl-peptidase IV (S09.003 in the MEROPS database), which cleaves after a proline residue when the residue two after the proline is also a proline (peptide-P/X-P-peptide). Again, these peptidases are not specific and the Bac peptides contain numerous possible cleavage sites that were not cleaved.

4.7 MINIMUM INHIBITORY CONCENTRATIONS

To determine the minimum inhibitory concentrations (MICs) of the purified peptides the radial diffusion assay MIC method was used. The more common micro-broth dilution method could not be used because of the limited amount of material purified. The details of the method are described in Section 3.1.7. A number of concentrations of the peptides were tested for antimicrobial activity using the radial diffusion plate assay method. For higher concentrations the resulting clearings were larger. The peptide concentrations were plotted on a log-scale against the clearing sizes on a linear scale, and straight lines were fitted.

From the equations of the lines, the MICs could be calculated using Equation 4.1. The MICs corresponded to the peptide concentration where the clearing size is zero, which is the x-intercept on the graph.

$$MIC = e^{\frac{-y\,int}{grad}} \qquad \text{Equation 4.1}$$

where y int is the y-intercept and grad is the gradient of the log-concentration versus clearing size plot.

e.g. For the MIC of OaBac5γ against *E. coli* O111, the equation of the line-of-best-fit was y = 0.9181x – 1.7055, so the MIC was calculated as follows:

$$MIC = e^{\frac{-y\,int}{grad}} = e^{\frac{-1.7055}{0.9181}} = 6.4\mu g/ml$$

The calculated MIC values and the limits of the associated 95% confidence intervals for the purified peptides Pa, Pb and Pc are summarized in Table 4.6. The limits of the 95% confidence intervals were calculated as described in Section 3.1.7. The raw data and example calculations are given in Appendix A1.2. The MICs were not determined for Pd and Pf because these samples were a mixture of peptides, not single pure peptides.

Table 4.6 - Minimum inhibitory concentrations of peptides purified from ovine neutrophil extract.

Organisms	NaCl conc (mM)	Minimum inhibitory concentration (μg/mL)		
		Pa/OaBac5	Pb/OaBac7.5$_{(32-60)}$	Pc/OaBac5γ
E. coli O111	0	3.48 (0.84, 5.77)	11.93 (NA, 17.71)	1.95 (1.87, 2.02)
	100	>25	11.93 (NA, 17.71)	22.15 (NA, 54.56)
S. aureus NCTC 4163	0	12.02 (NA, NA)	10.67 (NA, 18.63)	10.20 (1.27, 19.47)
	100	>30	>30	>125
C. albicans 3153A	0	>30	13.60 (NA, 21.92)	44.29 (NA, 77.61)
	100	>30	12.02 (NA, NA)	31.32 (NA, 64.12)

The values given in brackets are the limits of the 95% confidence intervals for the MICs. 'NA' means that the limits of the confidence intervals could not be calculated because the bounds did not cross the x-intercept.

Of the three peptides tested, OaBac5γ (Pc) was the most active against the test bacteria, and OaBac7.5$_{(32-60)}$ was the most active against the yeast. OaBac7.5 $_{(32-60)}$ retained its activity against *E. coli* and *C. albicans*, but not *S. aureus* at high salt concentrations, whereas the activity of OaBac5γ was impaired against both bacterial strains.

OaBac5 (Pa) had similar activity to OaBac5γ against the bacteria, but its MIC against the yeast could not be evaluated because there was not enough material to test higher concentrations. OaBac5 and OaBac5γ only differ by one amino acid so it is expected that their activity would be similar. OaBac5γ has a leucine residue instead of an arginine residue in OaBac5, which is a change from a positively charged amino acid to a hydrophobic one.

4.8 SEQUENCE ANALYSIS OF PROLINE/ARGININE-RICH PEPTIDES

All of the antimicrobial peptides purified from the second gel filtration fraction of the crude ovine neutrophil extract were proline/arginine-rich cathelicidins called bactenecins. As well as these, there are a number of other bactenecins that have previously been identified in sheep and other ruminants. The sequences of all the known proline/arginine-rich cathelicidin peptides are given in Table 4.7. These sequences were analysed and compared as described in Section 3.1.11.

Table 4.7 - Identification of repeats in the sequences of the proline/arginine-rich cathelicidin peptides.

Peptide	Reference	Repeat	Sequence	Reference
PR39	purified from porcine blood	(RXPP) (XXPP) (RXPP)	RRRPRPPYLPRP RPPP FFPP RLPP R**I**PP **G**FPP R**F**PP RFP	Agerberth *et al*, 1991
Bac4	predicted from bovine cDNA	$(XPXP)_2(XPRP)_2$	RRLHPQHQRFPRERP WPKP LSLP LPRP GPRP WPKP L	Scocchi *et al,*. 1998
OaBac6	predicted from ovine cDNA	$(XPXP)_2(XPRP)_2$	RRLRPRHQHFPSERP WPKP LPLP LPRP GPRP WPKP LPLP LPRP G**L**RP WKPL	Huttner *et al*, 1998
Bac5	purified from bovine blood	$(XRPP)_4$	RFRPPI RRPP IRPP F**Y**PP FRPP **I**RPP I**F**PP **I**RPP FRPP LGPFP	Frank *et al*. 1990
OaBac5	predicted from ovine cDNA	$(XRPP)_4$	RFRPPI RRPP IRPP FRPP FRPP **V**RPP IRPP FRPP FRPP IGPFP	Huttner *et al*, 1998
ChBac5	purified from caprine blood	$(XRPP)_4$	RFRPPI RRPP IRPP F**N**PP FRPP **V**RPP FRPP FRPP FRPP IGPFP	Shamova *et al*, 1999
Bac7	purified from bovine blood	$LP(XPRP)_3$	RRIRPRPPR LPRP RPRP LP FPRP GPRP IPRP LP FPRP GPRP IPRP LP FPRP GPRP IPRP	Frank *et al*. 1990
OaBac7.5	predicted from ovine cDNA	$LP(XPRP)_3$	RRLRPRRPR LP **R**PRP RPRP **R**PR**S** LP LPRP **Q**PR**R** IPRP IL LP **WRP**P RP**I**P **RPQ**P QPIP RWL	Huttner *et al*, 1998
OaBac11	predicted from ovine cDNA	$LP(XPRP)_3$	RRLRPRRPR LP **R**PRP RPRP **R**PR**S** LP LPRP **K**PRP IPRP LP LPRP RP**K**P IPRP LP LPRP RPR**R** IPRP LP LPRP RPRP IPRP LP LP**Q**P **Q**P**S**P IPRP L	Huttner *et al*, 1998

The repeats are shown by underlining. The residues that differ from the repeat pattern are indicated by bold-type.

Manual sequence analysis revealed that the proline/arginine-rich peptides all contain a repeating motif of four residues and larger repeated sequences made up of a combination of the smaller four-residue motifs. The motifs and repeats are highlighted in Table 4.7. In each case, the motif contains two prolines, one arginine and one varying residue. The order of these residues differs between the peptides. The varying residue is usually a hydrophobic amino acid such as I, L, F and V. The sequences were further analysed by generating hydrophobicity and polarity plots using the method described in Section 3.1.11. The plots are presented in Figure 4.7 and Figure 4.8.

The three Bac5 peptides contained two copies of a 16-residue repeat each made up of four of the four-residue motifs. These repeats were evident in the hydrophobicity and polarity plots of these peptides (Figure 4.7 and Figure 4.8). These plots also illustrated that the N-terminal end of the peptides were more polar, and the C-terminal ends were more hydrophobic.

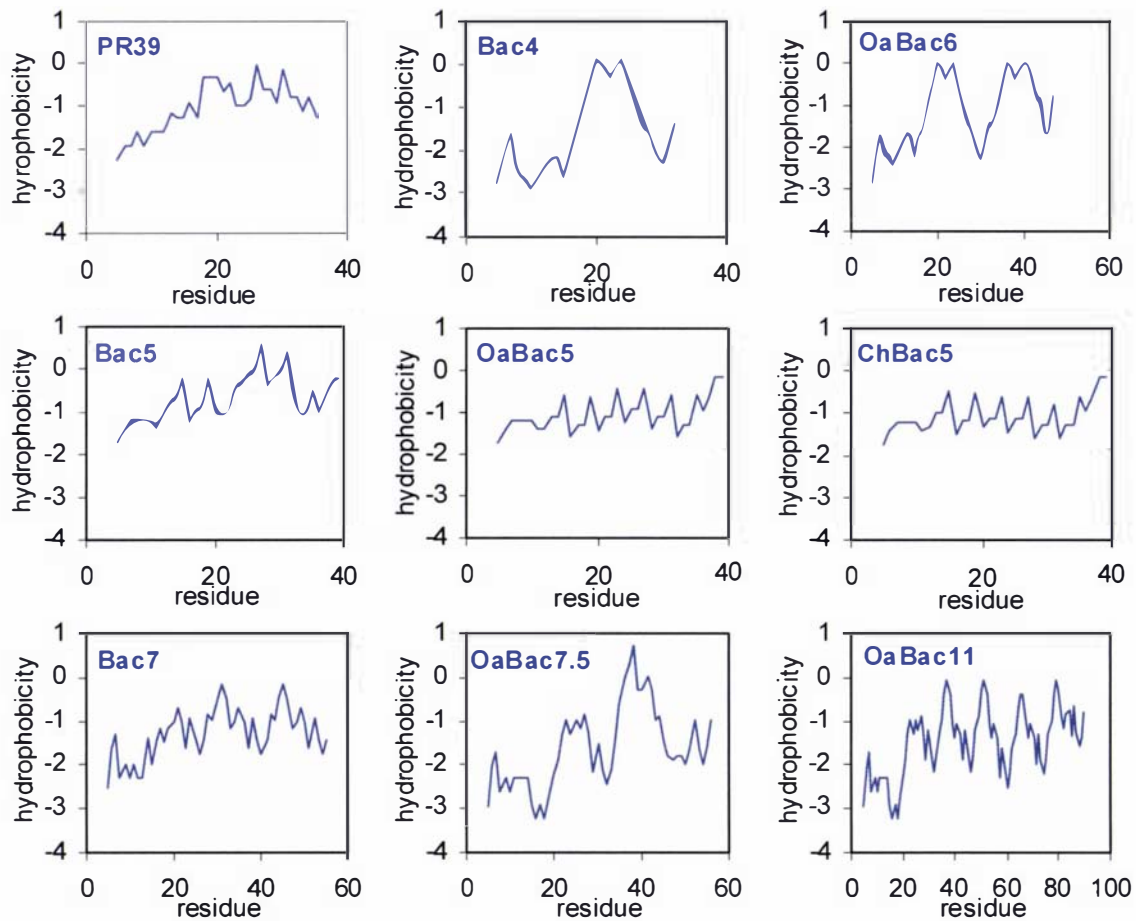

Figure 4.7 - Hydrophobicity plots of the proline/arginine-rich cathelicidin peptides.

The hydrophobic and polarity plots also highlighted differences in the sequences of the Bac5 peptides from the different species. Bovine Bac5 only differed from OaBac5 by 5 residues; however, two of these differing amino acids are arginine residues in the OaBac5 sequence but tyrosine and phenylalanine residues in the Bac5 sequence. This made Bac5 more hydrophobic and less cationic than OaBac5. Bac5 had comparable activity to OaBac5 against *E. coli*, but unlike OaBac5 it was not active against *S. aureus* (Gennaro *et al*, 1989).

The differences between OaBac5 and ChBac5 were less significant. These two sequences differ by only two amino acids. Only one of the substitutions alters the polarity; an arginine in OaBac5 is replaced with an asparagine in ChBac5. ChBac5 has similar activity to OaBac5, showing that this substitution does not alter the activity considerably (Shamova *et al*, 1999). However, like OaBac5α and OaBac5γ, ChBac5 is not inhibited by a salt concentration of 100mM NaCl.

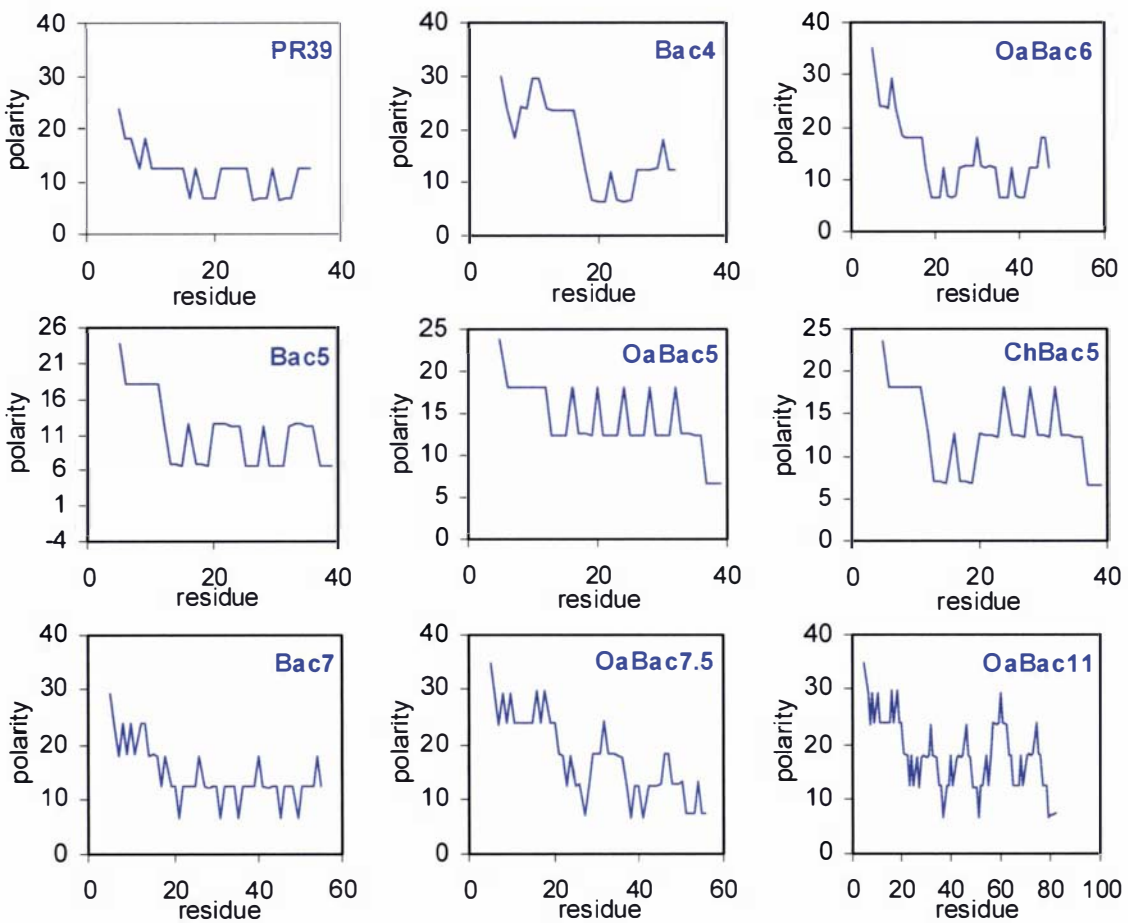

Figure 4.8 - Polarity plots of the proline/arginine-rich cathelicidin peptides.

Like the Bac5 peptides, OaBac6 also had two copies of a 16-residue repeat. However, its four-residue motif was different to that of the Bac5 peptides. Bovine Bac4 also contained the same 16-residue repeat as ovine OaBac6, but it had only one copy compared to OaBac6's two copies. Again, the plots showed a polar N-terminus, but the C-terminus was not as hydrophobic as that of the Bac5 peptides.

The last three peptides, Bac7, OaBac7.5 and OaBac11, had the same four-residue motif as Bac4 and OaBac6, but their repeat pattern was different. The 14-residue repeat started with the amino acids leucine and proline followed by three sets of the four-residue motif. Bac7 and OaBac7.5 contained three copies and OaBac11 contained six copies of this 14-residue repeat. In the case of Bac7, the repeats were perfect, whereas for the ovine peptides the sequence differed from that of the repeat numerous times. For OaBac7.5 there were two residues (LP) between the second and third repeats, which made the sequence even less uniform.

4.9 PURIFICATION OF ANTIMICROBIAL PEPTIDES USING CATIONIC EXCHANGE CHROMATOGRAPHY AND RP-HPLC

Cationic exchange chromatography was used as an alternative to the gel filtration purification step. This method separated the cationic molecules from the neutral and anionic molecules in the crude extract using the method described in Section 3.1.4. The crude extract was added to the cationic column and the non-cationic molecules were washed through the column with 25mM ammonium acetate solution. The cationic molecules were eluted from the column with 5% acetic acid. A salt gradient was not used to elute the cationic molecules because salt is detrimental to the activity of the peptides as shown in Section 7.2. A chromatograph of a typical run is shown in Figure 4.9.

The plate assay method was used to test the activity of the fractions. These results are summarised in Table 4.8. The molecules that did not bind to the column did not have antimicrobial activity, whereas the cationic fractions that were eluted from the column were active.

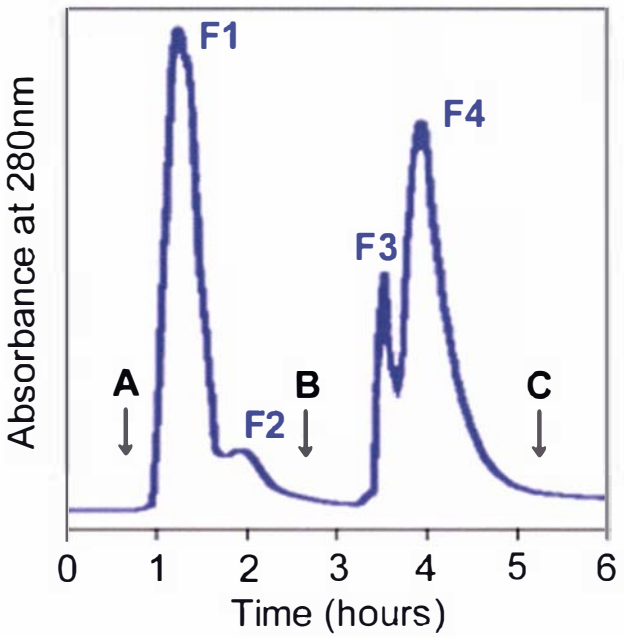

Figure 4.9 - Ion-exchange chromatograph for the addition of the ovine neutrophil crude extract to a weak cationic exchange column. The column was packed with Macro-Prep CM resin. At time 'A' the sample was added, at time 'B' the running buffer was changed from 25mM ammonium chloride to 10% acetic acid and at time 'C' the running buffer was changed to 20% ethanol.

Table 4.8 - Antimicrobial activity of typical ovine neutrophil extract cationic-exchange chromatography fractions against test organisms.

Test solution	Diameters of clearings on plate assays (mm)		
	E. coli O157:H7	*S. aureus* 1056 MRSA	*C. albicans* 3153A
positive control*	15	9	11
F1	no clearing	no clearing	no clearing
F2	no clearing	no clearing	no clearing
F3	6	6	5
F4	6	5	4
negative control**	no clearing	no clearing	no clearing

*** The positive controls were 1μg/mL polymyxin for *E. coli* O157:H7, 1μg/mL nisin for *S. aureus* 1056 MRSA, and 10μg/mL nystatin for *C. albicans* 3153A.**
**** The negative control was 0.01% acetic acid.**

The two cationic fractions, F3 and F4, were pooled together and RP-HPLC was used to separate them further. For this work a different HPLC technique was used than for the separation of the gel filtration fraction already described. The details of the methods used are given in Section 3.1.5. Despite the differences in the two methods, a similar separation pattern was seen and the peak activities were comparable to those of the proline/arginine-rich peptides already isolated.

One difference between the gel filtration F2 and the cationic fraction was that the components that came through in the void volume, when separating the proline/arginine-rich peptides using a gradient of 25-30% acetonitrile, also had antimicrobial activity. To separate these components a less hydrophobic gradient of 0-20% acetonitrile was used. Using this gradient, 36 peaks were separated. The chromatograph of a typical run is shown in Figure 4.10.

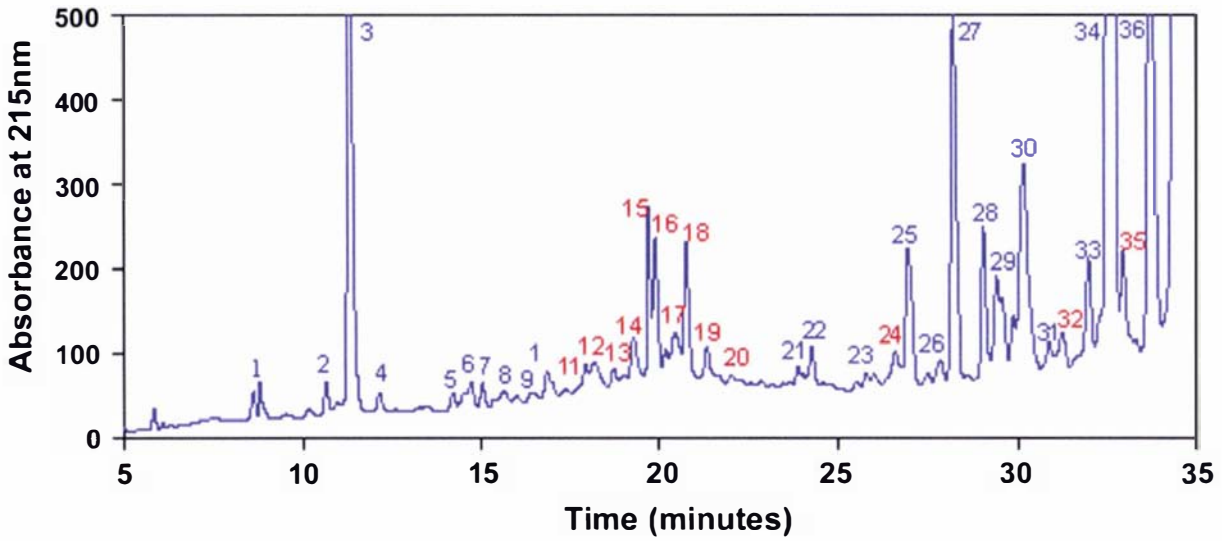

Figure 4.10 - RP-HLPC chromatograph of the cationic fraction (F3 and F4) of the ovine crude extract. Buffer A contained 5% acetonitrile and 0.1% trifluroacetic acid (TFA). Buffer B contained 95% acetonitrile and 0.1% TFA. A gradient of 0% to 20% of Buffer B over 30 minutes was used. The peaks with red labels had antimicrobial activity; the peaks with blue labels did not.

The plate assay method was used to test the activity of the 36 HPLC peaks collected. The results are summarised in Table 4.9. Of these, nine were active against both bacterial strains (*E. coli* O111 and *S. aureus* NCTC 4163). A further four peaks were active only against *E. coli* O111. None of the peaks displayed activity against the yeast (*C. albicans* 3153A). It is unknown whether the all of the components were inactive against yeast, or whether the concentrations tested were too low for the activity to be detected.

Of the active peaks, six were investigated further using mass spectroscopy. These peaks were chosen for mass spectroscopy because they had good activity against both the Gram-negative and Gram-positive test bacteria, and because they were well separated from the other peaks in the chromatograph. The determined molecular weights are given in Table 4.9. Most of the peaks had low molecular weights of 1-2kDa. Due to limited resources only two of the peaks, Peak 18 and Peak 24, were studied further using N-terminal sequencing.

Table 4.9 - Plate assay results and molecular weights of antimicrobial peptides isolated from the cationic fraction of ovine neutrophil extract.

Test solution	MWt (kDa)*	Diameter of clearings on plate assays (mm)	
		E. coli O111	*S. aureus* NCTC 4163
peak 11	-	3	-
peak 12	-	4	-
peak 13	-	3	-
peak 14	666.03, 1024.56	5	3
peak 15	1024.65	4	4
peak 16	-	4	4
peak 17	-	5	4
peak 18	3184.51	4	4
peak 19	1651.39	4	4
peak 20	-	4	4
peak 24	1126.06	6	5
peak 32	786.56, 1578.13	5	4
peak 35	-	3	-

*** The molecular weights were obtained experimentally (see Appendix A1.1 for mass spectra).**

Peak 18 was chosen for N-terminal sequencing because it was separated well from the other peaks and because it was the only peak of those tested that had a mass in the range expected for antimicrobial peptides (3-5kDa). The N-terminal sequence of Peak 18 was similar to part of the cathelin-like domain of cathelicidins. Peak 18 and SMAP29 prepropeptide are compared in Table 4.10.

It was previously thought that the cathelin-like precursor 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 recent study using recombinant human cathelin protein showed that the cathelin domain was able to inhibit the activity of the protease cathepsin L (Zhao *et al*, 1995b). This study also showed that, when cleaved, the cathelin domain displayed antimicrobial activity, whereas the full cathelicidin molecule did not. These findings suggested 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 yet it still inhibited the growth of the test bacteria. This shows that the complete cathelin domain is not required for antimicrobial activity.

Table 4.10 - Comparison of the N-terminus of cationic Peak 18 to the cathelin-like precursor of SMAP29.

Peptide	Source	Sequence	Reference
peak 18 N-terminal	purified from cationic fraction	LSLY-EAVLYAVDT :: : :::: :::	This work
SMAP29 prepropeptide	predicted from ovine cDNA	METQRASLSLGRRSLWLLLLGLVLASARAQALS-YREAVLRAVDQLNE KSSEANLYRLLELDPPPKQDDENSNIPKPVSFRVKETVCPRTSQQPAE QCDFKENGLLKECVGTVTLDQVGNNFDITCAEPQSVRGLRRLGRKIAH GVKKYGPTVLRIIRIAG	Huttner *et al*, 1998

The signal peptide is shown in red, the cathelin-like presequence in blue and the cationic antimicrobial peptide in black.

Table 4.11 - Comparison of the N-terminus of cationic Peak 24 to the signal peptide of T-cell surface glycoprotein CD4.

Peptide	Source	Sequence	Reference
peak 24 N-terminal	purified from cationic fraction	VLQLAL ::::::	This work
human T-cell surface glycoprotein CD4 precursor		MNRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKS IQFHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNFPLII KNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLE SPPGSSPSVQCRSPRGKNIQGGKTLSTWTCTVLQNQKKVEFKIDIVVL AFQKASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKS WITFDLKNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSGNLT LAEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKREK AVWVLNPEAGMWQCLLSDSGQVLLESNIKVLPTWSTPVQPMALIVLGG VAGLLLFIGLGIFFCVRCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQ KTCSPI	Maddon *et al*, 1986

The signal peptide is shown in red and the T-cell surface glycoprotein CD4 is shown in black.

The N-terminal sequence of Peak 24 was also determined. This peak was chosen for N-terminal sequencing because it had the most potent activity of the peaks collected from the cationic fraction even though it was one of the smallest peaks. The N-terminal sequence of Peak 24 matched part of the signal peptide of the T-cell surface glycoprotein CD4 precursor (Maddon *et al*, 1986). Peak 24 and human T-cell surface glycoprotein CD4 precursor are compared in Table 4.11. The fact that this fragment of the signal peptide displays antimicrobial activity shows that the signal peptide itself may also play a role in fighting infections.

4.10 OTHER PREDICTED CATHELICIDINS

Although numerous peptides were purified, this research did not find all seven of the cathelicidin peptides predicted from ovine DNA. Neither of the α-helical peptides, SMAP29 and SMAP34 were purified, nor was the dodecapeptide OaDode, nor one of the proline/arginine-rich peptides, OaBac6.

The undetected cathelicidins may have been present in their precursor forms and therefore were inactive, or they may have been in their active forms, but at concentrations too low to be detected by the antimicrobial activity testing method used. Another possibility is that the peptides were destroyed by the extraction process or by proteases present in the crude extract. Alternatively, they may not have been present in the crude extract. This could be the case if the peptides were not constitutively expressed, and instead are expressed only under certain circumstances, or if they were expressed in cells other than the neutrophils.

One possibility is that the undetected cathelicidins were still attached to their prosequences, which would make them inactive and, therefore, not detectable with the plate assay. Other groups have reported that it is necessary to add neutrophil elastase to the crude extract to cleave the proregion and release the active peptides (Shamova *et al*, 1999). However, for this research, the cleavage must have been carried out by neutrophil elastase naturally present in the crude extract because the extraction process did not include the addition of this enzyme. In the current work, the addition of human neutrophil elastase (ART Biochemicals) did not increase the activity or show any differences in the SDS-PAGE of the crude extract, so the cathelicidin processing was complete.

Another possibility is that some of the cathelicdins were destroyed during the extraction process, either by the process itself or by proteases present in the crude extract. One way to attempt to increase the number of isolated cathelicidins would be to boil the solution or to add protease inhibitors during the crude extraction process to stop peptide degradation. However, the neutrophil elastase present in the sample would be inhibited so the cathelicidins would be purified in their complete, inactive forms. Therefore, the addition of neutrophil elastase would be needed to process the cathelicidins and release the active peptides. This was undesirable for this project because it aimed to develop ways of using the components present in waste ovine blood, and the addition of elastase would be uneconomic in a large-scale process.

4.11 CONCLUSIONS

The objective of the work presented in this chapter was to purify and identify antimicrobial peptides from ovine blood. As hypothesised, the majority of the antimicrobial activity displayed by the ovine neutrophil crude extract was due to cathelicidin peptides. Previously seven cathelicidins had been predicted from ovine cDNA but only two variants of one of the predicted peptides had been purified from ovine blood. In this study numerous proline/arginine rich cathelicidins peptides were purified, including OaBac5, OaBacγ, OaBac7.5$_{(32\text{-}60)}$ and various truncates of OaBac11.

Many of the peptides purified were in truncated forms compared to the full peptides predicted from ovine cDNA. Peptidases capable of carrying out the cleavages were identified but these peptidases were not specific to the sites cleaved and could have resulted in many more truncations. Therefore, it is thought that the cleavage may have been carried out by peptidases that have not yet been characterised.

Even though many of the purified peptides were shorter than those predicted they still had antimicrobial activity against the three test organisms. This shows that the full molecules are not necessary for the antimicrobial activity. This property may contribute to the protection of the animal against pathogens, because even if the peptides are degraded by proteases secreted by the pathogens, the resulting truncates may still retain their antimicrobial function.

Although many of the predicted cathelicidins that had not previously been purified were isolated in this study, there were other predicted peptides that were not isolated. It is unclear whether these peptides were degraded during the extraction process or by proteases in the

extract, or whether they were not present in the crude extract because they are not constitutively expressed in the blood.

As well as these cathelicidin peptides, there were a number of other active components present in the crude extract. One component was identified as a fragment of the cathelin-like domain of cathelicidins. Recently, it was shown that the cathelin domain had antimicrobial activity (Zhao *et al*, 1995b). However, the peptide isolated in this study was only a small fragment of the cathelin domain, which showed again that the full molecule was not necessary for bioactivity.

Another of the purified peptides was possibly a fragment of T-cell surface glycoprotein CD4 precursor. This indicates further that the signal peptides and precursors of immune-related proteins may have a secondary role as antimicrobial agents.

CHAPTER 5
SPECTRUM OF ACTIVITY AND BACTERIAL MEMBRANE INTERACTIONS OF SYNTHETIC OVINE CATHELICIDINS

5.1 INTRODUCTION

The work presented in this chapter was concerned with the second objective of this research project, which was to determine the mechanism of action of ovine antimicrobial peptides. It was not practical to purify the necessary amounts of each of the individual peptides from the crude extract because each HPLC run yielded only approximately 30μg of each peptide and 5mg of each peptide was required for these tests. Instead, three peptides, OaBac5mini, OaBac7.5mini and SMAP29, were synthesised using the method described in Section 3.2.1. The peptide sequences are given in Table 5.1.

Table 5.1 - Sequences of synthetic ovine antimicrobial peptides used for this research.

Peptide	Sequence
OaBac5mini (N-terminal of Pa)	RFRPPIRRPPIRPPFRPPFRPPVR-NH_2
OaBac7.5mini (Pb)	RRIPRPILLPWRPPRPIPRPQPQPIPRWL-NH_2
SMAP29	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH_2

OaBac5mini is a truncated version of OaBac5, comprising the first 24 N-terminal residues. OaBac5 is a 51-residue, proline/arginine-rich peptide that was inferred based on a cDNA sequence (Huttner *et al*, 1998). Since then, three variants of OaBac5, as well as the originally predicted molecule, have been isolated from sheep neutrophils (Shamova *et al*, 1999)(Section 4.4). As shown in Section 4.8, 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, the truncated version of OaBac5, is made up of the six N-terminal residues, one copy of the 16-residue repeat, and the first two residues of the second repeat. Due to the large number of proline residues, the full-length molecule could not be synthesised. This truncation was chosen because it has been shown that the bovine peptide Bac7 retains its activity when truncated similarly (Sadler *et al*, 2002).

The second synthesised peptide was OaBac7.5mini, which is a truncated form of OaBac7.5. Like OaBac5, OaBac7.5 is also rich in proline and arginine and was predicted from ovine cDNA (Huttner *et al*, 1998). However, OaBac7.5, which was predicted to be 60-residues

long, has only been isolated from ovine neutrophil as the 29 amino acid C-terminal peptide, OaBac7.5$_{(32-60)}$ (Section 4.5). Therefore, it was this purified form that was synthesised for these tests.

The final peptide synthesised for these tests was SMAP29. SMAP29 is a very potent α-helical peptide (Skerlavaj *et al*, 1999) that was predicted from ovine cDNA (Mahoney *et al*, 1995; Bagella *et al*, 1995), but has not yet been isolated from ovine cells. This peptide was chosen for these tests, as a comparison to the other two peptides, because it is an α-helical peptide, whereas the others are proline/arginine-rich peptides.

The first objective of the work presented in this chapter was to determine the spectra of activity of the three test peptides. It has been shown previously by other research groups that SMAP29 has potent broad-spectrum activity (Skerlavaj *et al*, 1999). However, the spectra of activity of OaBac5mini and OaBac7.5mini, or their parent molecules, have not been determined before.

The second objective of the work presented in this chapter was to investigate the secondary structures of the peptides under different conditions. It was thought that the peptides would have random structures in aqueous solutions and form amphipathic structures in membrane-like conditions. All cationic antimicrobial peptides have a large number of hydrophobic residues that allow them to fold into structures with separate hydrophobic and cationic regions. This enables the peptides to insert themselves into bacterial membranes, with their hydrophobic region interacting with the oily membrane interior, while their cationic region interacts with anionic phospholipid head groups (Ganz, 1999).

The final objective of the work presented in this chapter was to investigate the basis of the synthesised peptides' antimicrobial activities by assessing the way they interact with bacteria. As reviewed in Section 2.6.2, it is believed that the steps involved in the mechanism of action of cationic antimicrobial peptides against Gram-negative bacteria are (Zasloff, 2002):

1) The positively charged peptides interact with negatively charged lipopolysaccharide (LPS) at the divalent cation binding sites on the outer surface of Gram-negative bacteria.
2) The peptides form pores in the outer bacterial membrane, which increases the permeability of the membrane.
3) The peptides pass across the outer bacterial membrane by a process termed "self-promoted uptake" and interact with the cytoplasmic membrane.

4) The peptides either insert into the cytoplasmic membrane, resulting in the loss of the proton gradient and leakage of essential molecules, or they translocate across the cytoplasmic membrane and interact with target molecules within the cytoplasm, such as DNA, RNA and proteins.

Each step in this proposed mechanism of action is illustrated in Figure 5.1. The capabilities of the peptides to carry out each of these steps were evaluated experimentally.

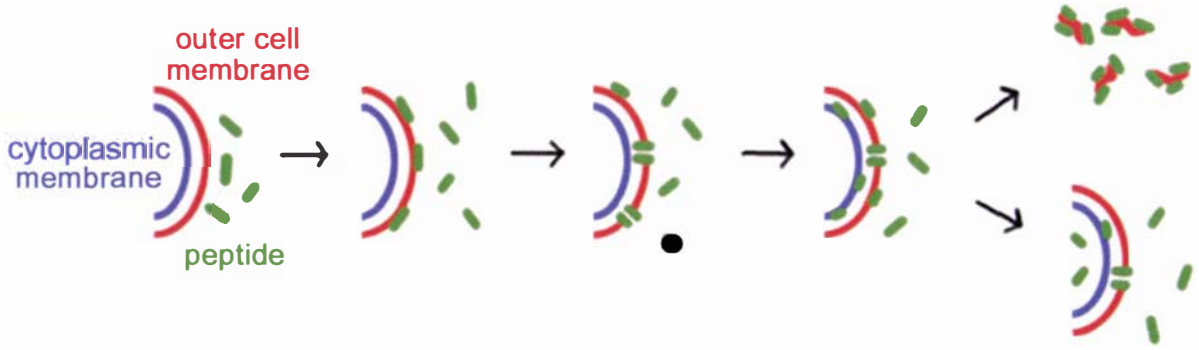

Figure 5.1 - Schematic diagram showing the proposed mechanism of action of antimicrobial peptides against Gram-negative bacteria.

5.2 MINIMUM INHIBITORY CONCENTRATIONS

The minimum inhibitory concentrations (MICs) of the three test peptides were determined using the modified micro-broth dilution method described in Section 3.2.2 (Wu and Hancock, 1999a). The test organisms included numerous Gram-negative and Gram-positive bacteria as well as two yeast strains. These organisms were chosen because they are either food pathogens or are clinically significant.

Five runs were carried out, each containing duplicates of each sample. In all cases, at least seven of the ten recorded values for the MIC of each peptide against each organism were the same. The MIC values varied from the mode values by only a single two-fold dilution. The variations were seen both between duplicates in the same run and between runs. The MICs are reported as the geometric means plus or minus the standard error in Table 5.2. The raw data is given in Appendix A2.1 and example calculations are given in Appendix A2.2.

Table 5.2 - Minimum inhibitory concentrations (MIC) of synthetic ovine antimicrobial peptides against various microorganisms.

Organism	MIC (μg/mL)		
	SMAP29	OaBac5mini	OaBac7.5mini
Gram-negative bacteria			
Escherichia coli O111	1.74 (1.45, 2.09)	2.14 (1.87, 2.46)	13.0 (10.6, 16.0)
E. coli UB1005 (rough K-12 strain)	0.13 (0.12, 0.15)	0.13 (0.12, 0.15)	6.96 (5.81, 8.35)
E. coli DC2 (antibiotic-supersusceptible mutant)	0.11 (0.09, 0.13)	0.12 (0.10, 0.13)	1.74 (1.45, 2.09)
E. coli O157:H7	2.00 (2.00, 2.00)	8.00 (6.53, 9.80)	32.0 (32.0, 32.0)
Salmonella typhimurium 14028s	0.23 (0.20, 0.27)	0.47 (0.41, 0.53)	29.9 (26.1, 34.2)
S. typhimurium MS4252S (*phoPQ* mutant; defensin supersusceptible)	0.12 (0.10, 0.13)	0.13 (0.12, 0.13)	1.62 (1.32, 2.00)
Pseudomonas aeruginosa PAO1	4.00 (4.00, 4.00)	4.29 (3.74, 4.91)	18.4 (15.3, 22.0)
P. aeruginosa Z61 (antibiotic-supersusceptible mutant)	0.93 (0.81, 1.07)	7.46 (6.52, 8.55)	32.0 (32.0, 32.0)
Gram-positive bacteria			
Staphylococcus aureus NCTC 4163	1.07 (0.94, 1.23)	29.9 (26.1, 34.2)	64.0 (64.0, 64.0)
S. aureus MRSA R147	0.50 (0.50, 0.50)	64.0 (64.0, 64.0)	36.8 (30.7, 44.1)
S. aureus 1056 MRSA	4.00 (4.00, 4.00)	18.4 (15.3, 22.0)	>64
S. epidermidis clinical isolate	0.29 (0.24, 0.34)	17.2 (15.0, 19.6)	32.0 (32.0, 32.0)
Enterococcus faecalis ATCC 29212	2.00 (2.00, 2.00)	32.0 (32.0, 32.0)	59.7 (52.1, 68.4)
Yeast			
Candida albicans 105	2.14 (1.87, 2.46)	27.9 (2302, 33.4)	64.0 (64.0, 64.0)
C. albicans 3153A	4.29 (3.74, 4.91)	19.7 (16.0, 24.2)	>64

The MICs are expressed as the geometric means of five runs, each with duplicate samples. The values in the brackets are the lower and upper limits of the 95% confidence intervals of the means. The raw data is given in Appendix A2.1 and example calculations are given in Appendix A2.2.

The potent, broad-spectrum activity of SMAP29 is already well documented (Skerlavaj *et al*, 1999; Brogden *et al*, 2001), but the activities of OaBac5mini and OaBac7.5mini have not been investigated previously. These results confirmed that SMAP29 had potent activity against all organisms tested (MICs of 0.1 to 4.3 μg/mL). The proline/arginine-rich peptides were generally less active than SMAP29.

OaBac5mini had potent activity against the Gram-negative bacteria (0.1 to 8 μg/mL), but comparatively weaker activity against the Gram-positive bacteria and the yeast *Candida albicans* (17.1 to 64 μg/mL). As expected, the truncated OaBac5mini had similar antimicrobial activity to that previously determined for the purified full peptide in Section 4.7. This result confirms that the full molecule was not necessary to retain the antimicrobial activity.

When compared to results reported for the activity of Bac5, a bovine proline/arginine-rich peptide analogue of OaBac5, OaBac5mini was more active against both Gram-negative and Gram-positive bacteria (Gennaro *et al*, 1989). OaBac5 and Bac5 differ in only five of their 43 residues, and are 88% homologous (determined using the online EMBL-EBI ClustalW tool at http://www.ebi.ac.uk/clustalw/ index.html). Two of these differing amino acids are arginine residues in the OaBac5 sequence, but tyrosine and phenylalanine residues in the Bac5 sequence. This makes Bac5 more hydrophobic and less cationic than OaBac5, which may account for the observed differences in activity.

OaBac7.5mini had relatively weak activity against all the test organisms except for the antibiotic super-susceptible strains *E. coli* DC2 (weak outer membrane) and *S. typhimurium* MS4252S (a *phoPQ* mutant), and the rough LPS strain *E. coli* UB1005. The activity of the synthetic OaBac7.5mini compared to the same peptide purified from ovine blood, shown in Section 4.7, was similar for *E. coli* O111, but greatly reduced for *S. aureus* NCTC 163 and *C. albicans* 3152A. This may be because different antimicrobial activity testing techniques were used.

The activity of the complete OaBac7.5 peptide has not been investigated. However, its bovine analogue (64% homologous), Bac7, has similar activity against Gram-negative bacteria, but is not as active against Gram-positive bacteria, compared to OaBac7.5mini (Gennaro *et al*, 1989).

5.3 CIRCULAR DICHROISM SPECTROSCOPY

The circular dichroism (CD) spectra were obtained for each test peptide in three different solutions using the method described in Section 3.2.3. Three different solutions were used to compare the structures of the peptides in different conditions. To represent aqueous, hydrophobic and membrane-mimicking conditions, phosphate buffer, 50% trifluroethanol (TFE) and liposomes were used respectively. The CD spectra are given in Figure 5.2.

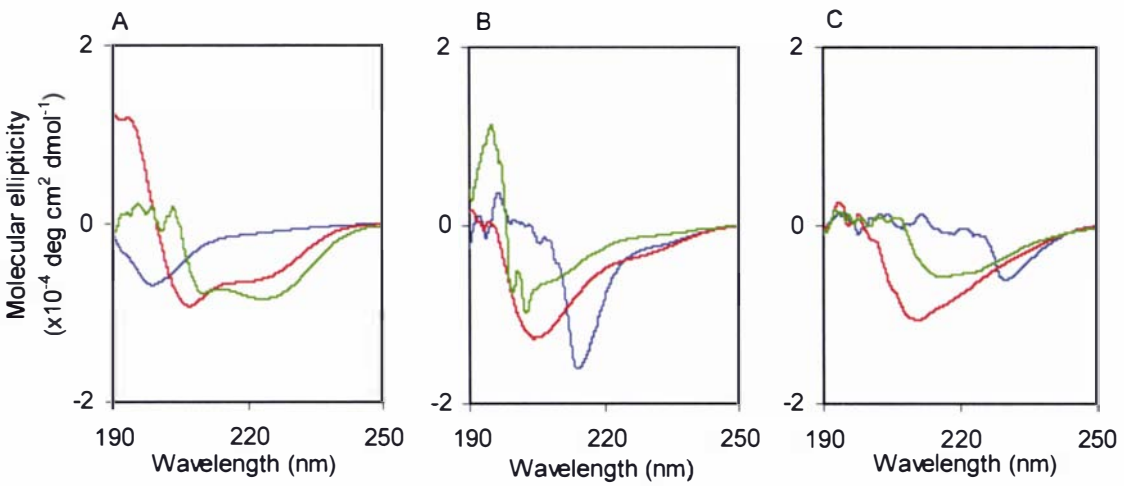

Figure 5.2 - Circular dichroism spectra of 25mM synthetic ovine antimicrobial peptides. (A) shows SMAP29, (B) shows OaBac5mini, and (C) shows OaBac7.5mini in the solutions 25mM phosphate buffer (—), 50% 2,2,2-trifluroethanol (—) and 10mM lysoPC/lysoPG (—).

The CD spectra of SMAP29 in aqueous buffer had a negative band at approximately 200 nm. This indicates that the structure was random. However, in 50% TFE (hydrophobic conditions), and 10 mM lyso-PC/lyso-PG (anionic membrane-mimicking conditions), SMAP29 had clear minima at approximately 206 and 230 nm. These are characteristics found in α-helices. This confirms that SMAP29 adopts an α-helical structure in these environments. These results are very similar to those previously obtained by another research group (Tack *et al*, 2001).

The structures of OaBac5mini and OaBac7.5mini were more difficult to assess based on their CD spectra. Both peptides appeared to be typical of a polyproline type II helix under membrane mimicking conditions with a minimum at 202 nm (Raj and Edgerton, 1995; Falla *et al*, 1996). OaBac5mini had more pronounced spectra than OaBac7.5mini. This was possibly because OaBac5mini may contain additional contributions from other turn structures, which tend to broaden the minimum that defines the polyproline type II helix. Although the

structures of these proline/arginine-rich peptides were difficult to determine, the results show that the peptides have different conformations in each of the different solvents. The wavelength shift of the minimum may have been related to the change in solvent polarity (water->TFE->lipid) amongst the different solutions.

5.4 LPS BINDING ASSAY

The first step in the interaction of antimicrobial peptides with Gram-negative bacteria is thought to be the binding of the cationic peptides to the anionic lipid A base of lipopolysaccharides (LPS) on the surface of the outer membrane of the cells. It has previously been shown that SMAP29 binds to LPS. SMAP29 has two sites that LPS can bind to cooperatively (Tack *et al*, 2001). However, the LPS binding abilities of the other two test peptides have not been investigated.

To determine if the test peptides were able to bind to bacterial LPS, their ability to displace dansyl polymyxin B (DPX) from LPS was examined using the method described in Section 3.2.4. A schematic diagram that shows the principle of the assay is given in Figure 5.3. DPX is a fluorescing compound that fluoresces only in its bound state. Initially the LPS solution is saturated with DPX so that the maximum amount of DPX is bound and maximum fluorescence occurs. When a test peptide is added to this LPS/DPX solution, if the test peptide is able to displace some of the DPX, then the fluorescence decreases because less DPX is in its bound state.

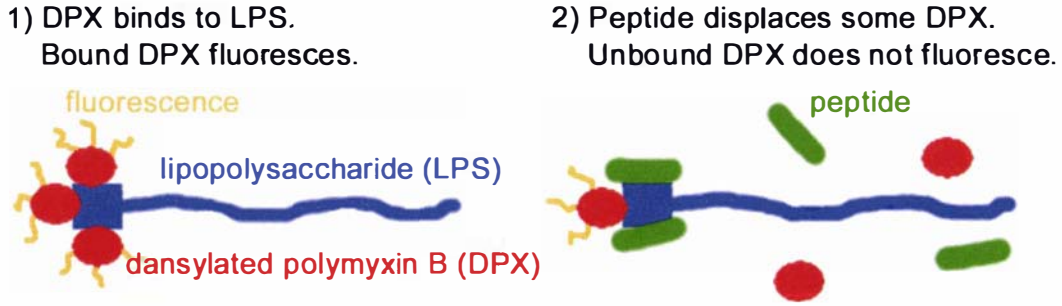

Figure 5.3 - Schematic diagram showing the mechanism involved in the lipopolysaccharide binding assay.

A graph of the fluorescence over time for a typical LPS binding assay run for SMAP29 is shown in Figure 5.4. The initial fluorescence was allowed to stabilise and was recorded. Then 1μg of SMAP29 was added to the 2mL volume to give a final concentration of SMAP29

of 0.5μg/mL. When the SMAP29 was first added this caused a spike in the fluorescence because of the presence of the pipette tip in the sample. Once the fluorescence stabilised the reading was recorded and then another 1μg aliquot of SMAP29 was added. This process was repeated six times.

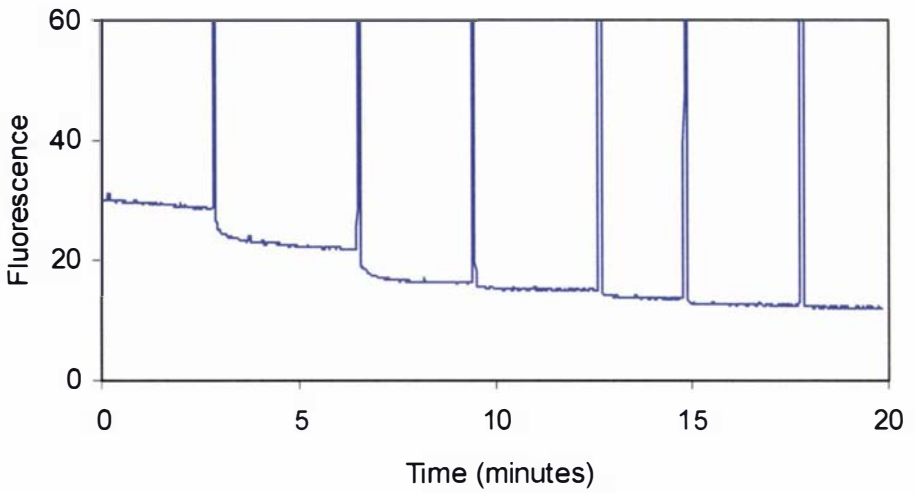

Figure 5.4 - A typical run showing the changes in the fluorescence of dansyl polymyxin B due to the addition of SMAP29. Each spike represents the addition of an aliquot of 1μg of SMAP29. This increased the total SMAP29 concentration in the sample by 0.5μg/mL with each addition.

The data collected from the experimental runs were then processed and analysed. The fraction of the DPX fluorescence that was inhibited at each concentration of SMAP29 was calculated using Equation 5.1.

$$F_{inhib} = \frac{F_{int} - F_n}{F_{int}} \qquad \text{Equation 5.1}$$

where F_{inhib} is the fraction of the fluorescence inhibited, F_{int} is the initial fluorescence reading before any SMAP29 was added, and F_n is fluorescence reading at concentration n.

e.g. After the first addition of SMAP29 (Table 5.3)

$$F_{inhib} = \frac{F_{int} - F_n}{F_{int}} = \frac{28.6 - 22.0}{28.6} = 0.23$$

The results of the calculations of the fraction of fluorescence inhibited for various SMAP29 concentrations for a typical SMAP29 run are summarised in Table 5.3. The inverse of the SMAP29 concentration and the inverse of the fraction of fluorescence inhibited were also calculated. From this, the inverse of the peptide concentration was plotted against the inverse

of the fraction of fluorescence inhibited according to the Lineweaver-Burke plot method. The graph of the SMAP29 concentration versus the fraction of fluorescence inhibited and the double reciprocal plot of these parameters are given in Figure 5.5.

Table 5.3 - Data collected and calculated for the change in dansyl polymyxin B fluorescence due to the addition of SMAP29 in a typical run.

SMAP29 total added (μg)	SMAP29 concentration (μg/mL)	1/SMAP29 concentration	Fluorescence reading	Fraction of fluorescence inhibited	1/fraction inhibited
0	0.00		28.6	0.00	
1	0.49	2.02	22.0	0.23	4.33
2	0.99	1.01	16.4	0.43	2.34
3	1.48	0.67	15.1	0.47	2.12
4	1.98	0.51	13.9	0.51	1.95
5	2.47	0.41	12.6	0.56	1.79
6	2.96	0.34	12.2	0.57	1.74

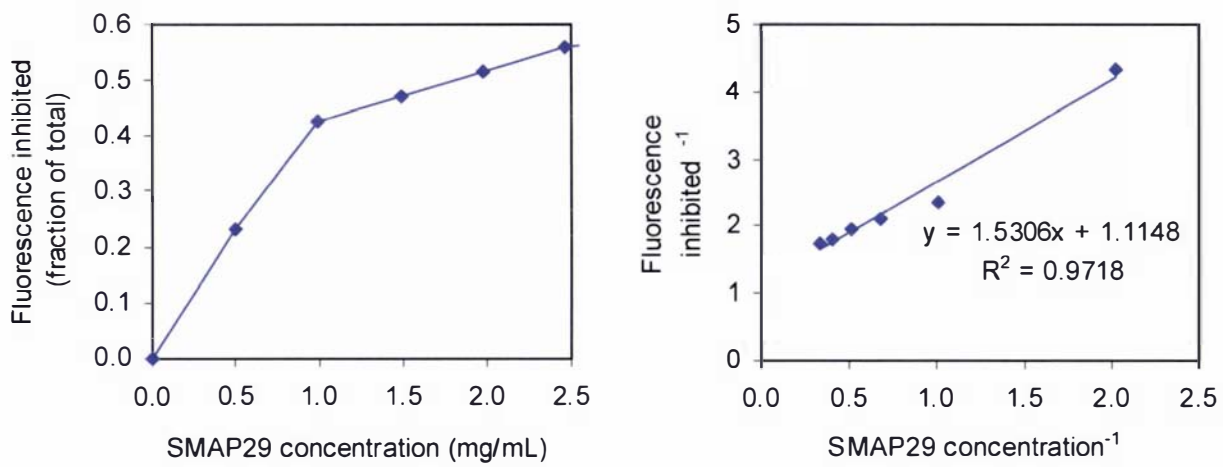

Figure 5.5 - Lineweaver-Burke plot for a typical run of the SMAP29-LPS binding assay.

From the equation of the line on the double reciprocal plot, two parameters, I_{max} and I_{50} were calculated. I_{max} is the maximum percentage of DPX that could be displaced by the peptides and I_{50} is the concentration of peptide required to achieve half the of its maximum DPX displacement. These factors were calculated using Equation 5.2 and Equation 5.3 respectively.

$$I_{max} = \frac{100}{\text{y-int}} \qquad \text{Equation 5.2}$$

where I_{max} is the maximum percentage of DPX displaced and y-int is the y-intercept on the Lineweaver-Burke plot.

e.g. For the typical SMAP29 run shown in Table 5.3 and Figure 5.5, the equation of the line was y = 1.5306x + 1.1148. Therefore:

$$I_{max} = \frac{100}{\text{y-int}} = \frac{100}{1.1148} = 89.7\,\%$$

$$I_{50} = \frac{-1}{\text{x-int}}$$ Equation 5.3

where I_{50} is the concentration of peptide required to displace half the maximum percentage of DPX displaced and x-int is the x-intercept on the Lineweaver-Burke plot.

e.g. For the typical SMAP29 run:

$$\text{x-int} = \frac{-(\text{y-int})}{\text{gradient}} = \frac{1.1148}{1.5306} = -0.7283$$

$$I_{50} = \frac{-1}{\text{x-int}} = \frac{-1}{-0.7283} = 1.37\ \mu g/mL$$

The I_{max} and I_{50} for each of the test peptides are given in Table 5.4. These values are the means of three runs and the values in the brackets are the limits of the 95% confidence intervals for the means. The raw data and calculations are given in Appendix A2.3 and Appendix A2.4, respectively.

Table 5.4 - The ability of synthetic ovine peptides to bind to *E. coli* lipopolysaccharide (LPS) using the dansyl polymyxin B (DPX) displacement assay.

Peptide	I_{max} (%)	I_{50} (μg/mL)
SMAP29	82.6 (71.9, 93.3)	0.99 (0.90, 1.08)
OaBac5mini	55.3 (48.8, 61.8)	3.58 (1.54, 5.62)
OaBac7.5mini	55.2 (34.5, 75.9)	4.84 (3.66, 6.02)

I_{max} is the percentage of DPX displaced relative to the total DPX bound.
I_{50} is the concentration of peptide required to give half the maximum DPX displacement.
Data are the means of three runs. The values in the brackets are the limits of the 95% confidence intervals for the means.

Of the three peptides, SMAP29 displaced the most DPX and caused 50% of its maximal displacement at the lowest concentration; therefore it had the highest affinity for LPS. For OaBac5mini and OaBac7.5mini, there were no significant differences in the mean I_{max} and I_{50} values owing to the large variation between triplicates. The calculations in Appendix 2.4 showed that p-values were 0.4966 and 0.1831 for I_{max} and I_{50} respectively. However, the I_{50} of OaBac5mini was lower than that of OaBac7.5mini in each run, so the trends were consistent between runs.

The greater ability of SMAP29 to bind to LPS, compared to the other two peptides, may be because it contains more cationic residues (ten) compared to the other two peptides (eight). However, this enhanced LPS binding did not correlate with enhanced activity against Gram-negative bacteria – these bacteria have LPS on their membranes. OaBac5mini had similar activity to SMAP29 against Gram-negative bacteria, even though SMAP29 has a much higher affinity for LPS. OaBac5mini was less active than SMAP29 against Gram-positive bacteria, which lack LPS on their outer surface. This would suggest therefore that the ability of the peptides to bind to LPS was not an important factor in determining the potency of their antimicrobial activity.

5.5 OUTER MEMBRANE PERMEABILISATION

After binding to the LPS on the outer membrane surface, it was expected that the cationic peptides would adopt amphipathic structures to adapt to the specific conditions at the membrane-water interface. The insertion of a large number of positive charges at this interface would disrupt surface electrostatics. This would lead to increased outer membrane permeability and the passage of peptide molecules through this membrane in a process termed "self-promoted uptake" (Hancock, 2001b).

To test whether the peptides increased the permeability of the outer membrane, their ability to induce the uptake of 1-N-phenylnapthylamine (NPN) by the bacterial cells was monitored using the method described in Section 3.2.5. A schematic diagram illustrating the principle of this method is given in Figure 5.6. NPN is a small hydrophobic molecule that is normally excluded by the outer membrane of Gram-negative bacteria. When NPN partitions into the bacterial outer membrane it fluoresces. This indicates that the permeabilisation of the outer membrane has occurred.

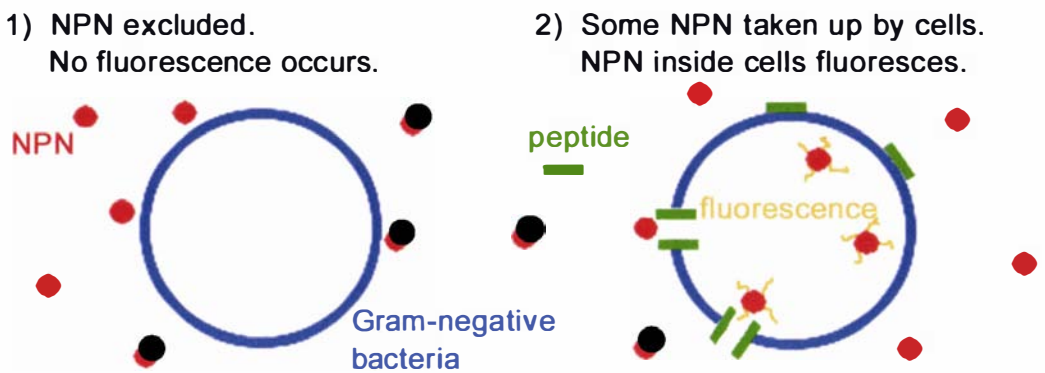

Figure 5.6 - Schematic diagram showing the mechanism involved in the 1-N-phenylnapthyl-amine (NPN) uptake assay.

For each peptide concentration, the initial fluorescence reading of the *E. coli* UB1005 culture mixed with NPN was recorded. This initial reading increased slightly throughout the day as the culture aged and the cell membranes became weaker. After the addition of the antimicrobial peptide the fluorescence was allowed to stabilise, then the reading was recorded. The data collected for a typical run with SMAP29 are given in Table 5.5.

Table 5.5 - Data collected and calculated for change in 1-N-phenylnapthylamine (NPN) fluorescence due to the addition of SMAP29 for a typical run.

SMAP29 concentration (μg/mL)	Initial fluorescence reading	Final fluorescence reading	Change in fluorescence	NPN uptake* (%)
0.0	22.5	22.5	0.0	0.0
0.1	21.3	26.7	5.40	3.4
0.2	30.6	55.0	24.40	15.3
0.3	28.1	50.2	22.10	13.8
0.4	28.3	57.2	28.90	18.1
0.5	50.6	119.8	69.20	43.3
1.0	36.0	122.3	86.30	53.9
2.0	45.0	156.2	111.20	69.5
4.0	56.4	184.0	127.6	79.8

*** The percentage of NPN taken up is compared to that caused by 4μg/mL polymyxin B.**

Since the readings varied between runs, the change in fluorescence due to the addition of the test peptides was compared to the change in fluorescence due to the addition of 4μg/mL polymyxin B using Equation 5.4 and Equation 5.5.

$$\Delta F_{pb} = F_{pb(final)} - F_{pb(intial)} \qquad \text{Equation 5.4}$$

where $F_{pb(initial)}$ is the background fluorescence reading before the polymyxin B was added, and $F_{pb(final)}$ is the fluorescence measurement after the addition of 4μg/mL polymyxin B.

e.g. For the typical run given in Table 5.5, the polymyxin B standard was calculated as follows:

$$F_{pb(intial)} = 22 \text{ and } F_{pb(final)} = 182$$

$$\Delta F_{pb} = F_{pb(final)} - F_{pb(intial)} = 182 - 22 = 160 \text{ fluorescence units}$$

$$\% \text{ NPN uptake} = \frac{\Delta F_n}{\Delta F_{pb}} \times 100 \qquad \text{Equation 5.5}$$

where ΔF_{pb} is the change in fluorescence caused by the addition of 4μg/mL polymyxin B and ΔF_n is the change in fluorescence caused by the addition of the peptide at concentration n.

e.g. For an SMAP29 concentration of 0.1 μg/mL in the typical run (Table 5.5):

$$\% \text{ NPN uptake} = \frac{\Delta F_n}{\Delta F_{pb}} \times 100 = \frac{5.4}{160} \times 100 = 3.4\%$$

For a typical SMAP29-NPN assay run, the percentage of NPN uptake compared to that of polymyxin B is summarised in Table 5.5. As expected the amount of NPN taken up by the cells increased with increasing peptide concentration. The addition of 4μg/mL SMAP29 caused less NPN to be taken up into the cells than that caused by the addition of 4μg/mL polymyxin B.

The NPN uptake caused by each peptide is summarised in Figure 5.7. The means of three runs and their associated 95% confidence intervals are plotted. The raw data and calculations are given in Appendix A2.5. The analysis of variance, which is reported in Appendix A2.6, showed that the probability of the mean MICs for each peptide being the same and the probability of the mean MIC at different concentrations being the same were both less than 0.0001 so the results were very significantly different. There was variation between the runs because the stability of the culture was different on each day; however, the trends were the same for each run.

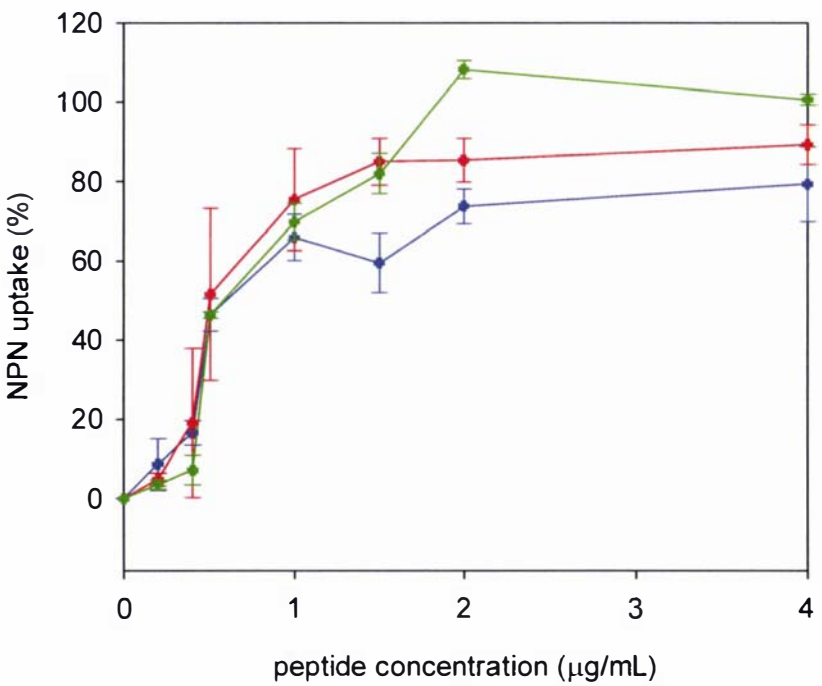

Figure 5.7 - Uptake of 1-N-phenylnapthylamine (NPN) by *E. coli* UB1005 cells caused by synthetic ovine peptides. The test peptides were SMAP29 (—), OaBac5mini (—) and OaBac7.5mini (—). The amount of NPN taken up is given as a percentage of the maximum NPN uptake caused by 4μg/mL polymyxin B. The points show the mean values of three runs and the bars show the 95% confidence intervals for the means.

All three peptides made the outer membrane of *E. coli* UB1005 cells more permeable to NPN. The peptide concentration that caused the half-maximal uptake of NPN was between 0.4 and 0.5μg/mL for each of the three peptides. For each peptide there was a large increase in permeability caused between peptide concentrations of 0.2 and 1μg/mL. At peptides concentrations of 2μg/mL the maximum permeability was reached and there appeared to be little further increase.

Of the three test peptides, OaBac7.5mini caused the highest total amount of NPN to be taken up, which was more than the total uptake caused by the control (4μg/mL polymyxin B); whereas SMAP29 caused the least amount of NPN to be taken up. This was unexpected since SMAP29 is a lot more active than OaBac7.5mini against this organism.

SMAP29 and OaBac5mini each caused small amounts of uptake of NPN at their MICs of 0.125μg/mL, but they required concentrations much higher than their MICs to cause a large increase in membrane permeability. This shows that a large amount of membrane permeabilisation is not necessary for the mechanism of action of these peptides. These peptides may form transient pores in the outer membrane to allow self-promoted uptake to occur, without requiring a large flux of molecules in and out of the cells to inhibit the organism.

In contrast, OaBac7.5mini caused substantial NPN uptake at concentrations well below its MIC of 8μg/mL against this organism. This means that the ability for this peptide to increase the permeability of the outer membrane of Gram-negative bacteria does not correlate with its antimicrobial activity. This property of OaBac7.5mini may be useful as it could be used in conjunction with other active molecules to increase the permeability of the outer membrane to allow the uptake of the other active molecules.

5.6 CYTOPLASMIC MEMBRANE DEPOLARISATION

After passing through the outer membrane of Gram-negative cells, antimicrobial peptides are then able to interact with the bacterial cytoplasmic membrane and depolarise and/or traverse this membrane (Hancock and Rozek, 2002). To assess the interaction of the peptides with the cytoplasmic membrane, the fluorescence of the dye 3,3-dipropylthiacarbocyanine ($DiSC_35$) was monitored using the method described in Section 3.2.6. A schematic diagram illustrating the principle of this assay is given in Figure 5.8. $DiSC_35$ is a cyanine dye that inserts into the

cytoplasmic membrane under the influence of the membrane potential gradient and quenches its own fluorescence. After the addition of a permeabilising peptide that disrupts membrane potential, the dye is released. This causes an increase in fluorescence.

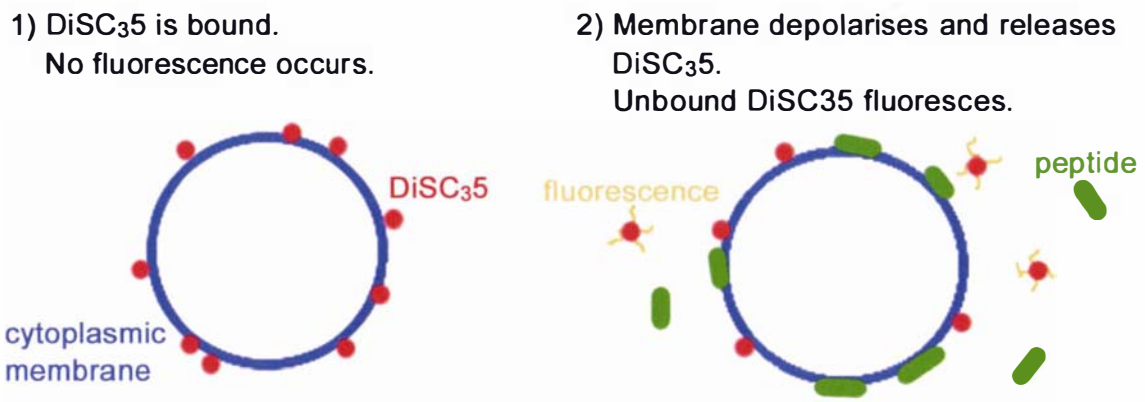

Figure 5.8 - Schematic diagram showing the mechanism involved in the 3,3-dipropylthiacarbocyanine ($DiSC_35$) assay.

For each peptide concentration the initial fluorescence reading of the *E. coli* DC2 culture mixed with $DiSC_35$ was recorded. This initial reading increased throughout the day as the culture aged and the inner cellular membranes became weaker. After the addition of the antimicrobial peptide the fluorescence was allowed to stabilise, then the final reading was recorded. The data collected for a typical run with SMAP29 is given in Table 5.6

Table 5.6 - Data collected and calculated for change in 3,3-dipropylthiacarbocyanine ($DiSC_35$) fluorescence due to the addition of SMAP29 for a typical run.

SMAP29 concentration (μg/mL)	Initial fluorescence reading	Final fluorescence reading	Change in fluorescence	$DiSC_35$ released* (%)
0.1	5.1	6.4	1.3	1.7
0.125	4.9	23.0	18.1	23.5
0.25	6.3	46.2	39.9	51.8
0.5	7.4	52.2	44.8	58.2
1.0	7.2	61.4	54.2	70.4
2.0	7.8	57.2	49.4	64.2
3.0	8.4	60.1	51.7	67.1
4.0	9.6	64.4	54.8	71.2

*The percentage of $DiSC_35$ released is compared to that caused by 4μg/mL gramicidin S.

Since the readings varied between runs, the change in fluorescence due to the addition of the test peptides was compared to the change in fluorescence due to the addition of 4μg/mL gramicidin S using Equation 5.6 and Equation 5.7. Gramicidin S was chosen for the

comparison since it is known to cause substantial depolarisation of the cytoplasmic membrane.

$$\Delta F_{gs} = F_{gs(final)} - F_{gs(intial)}$$ Equation 5.6

where $F_{gs(initial)}$ is the background fluorescence reading before the gramicidin S was added, and $F_{gs(final)}$ is the fluorescence measurement after the addition of 4μg/mL gramicidin S.

e.g. For the typical run given in Table 5.6, the gramicidin S standard was calculated as follows:

$$F_{gs(intial)} = 4.5 \text{ and } F_{gs(final)} = 81.5$$

$$\Delta F_{gs} = F_{gs(final)} - F_{gs(intial)} = 81.5 - 4.5 = 77 \text{ fluorescence units}$$

$$\%\ DiSC_3 5 \text{ released} = \frac{\Delta F_n}{\Delta F_{gs}} \times 100$$ Equation 5.7

where ΔF_{gs} is the change in fluorescence caused by the addition of 4μg/mL gramicidin S and ΔF_n is the change in fluorescence caused by the addition of the peptide at concentration n.

e.g. For an SMAP29 concentration of 0.1μg/mL in the typical run (Table 5.6):

$$\%\ DiSC_3 5 = \frac{\Delta F_n}{\Delta F_{gs}} \times 100 = \frac{1.3}{77} \times 100 = 1.7\%$$

For a typical SMAP29 run, the percentage of $DiSC_3 5$ released compared to that of gramicidin S is summarised in Table 5.6. The amount of $DiSC_3 5$ released from the cells due to the addition of 4μg/mL SMAP29 was substantially less than that released by the control 4μg/mL gramicidin S.

The results for each test peptide from the $DiSC_3 5$ assay are shown in Figure 5.9. The means of three runs and their associated 95% confidence intervals are plotted. The raw data and calculations are given in Appendix A2.7. The analysis of variance, which is reported in Appendix A2.8, showed that the probability of the mean MICs for each peptide being the same, and the probability of the mean MIC at different concentrations being the same, were both less that 0.0001 so the results were very significantly different. There was variation between the runs because the stability of the culture was different each day; however, the trends were the same for each run.

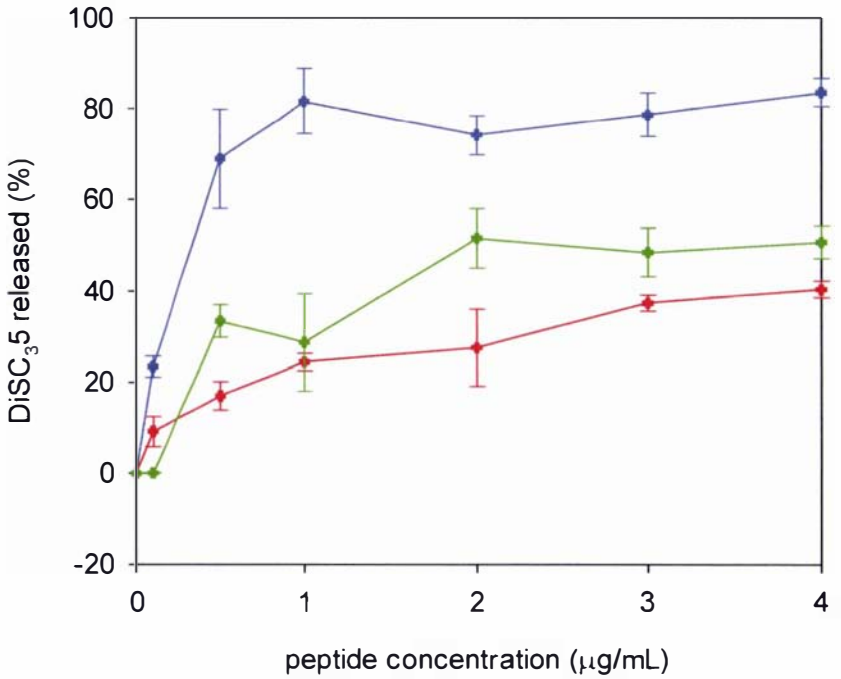

Figure 5.9 - Release of 3,3-dipropylthiacarbocyanine ($DiSC_35$) dye from the cytoplasmic membrane of *E. coli* DC2 cells caused by synthetic ovine peptides. The test peptides were SMAP29 (—), OaBac5mini (—) and OaBac7.5mini (—). The amount of $DiSC_35$ released by the peptides is given as a percentage of the maximum $DiSC_35$ released by 4μg/mL gramicidin S. The points show the mean values of three runs and the bars show the 95% confidence intervals for the means.

For all three peptides some $DiSC_35$ was released. This means that the peptides caused some depolarisation of the cytoplasmic membrane. For this to happen, the peptides must have interacted with the cytoplasmic membrane. This confirms that the peptides traversed the outer membrane, as predicted from the results of the outer membrane permeabilisation assay in Section 5.5.

SMAP29 caused depolarisation of the cytoplasmic membrane at its MIC of 0.125μg/mL, and had a maximum depolarisation of 83% of that of 4μg/mL gramicidin S. There was a rapid increase in the amount of depolarisation caused by SMAP29 between the concentrations of 0 and 0.5μg/mL, but there was little increase at concentrations higher than 0.5μg/mL, which shows that concentrations higher than this have no extra effect. This depolarisation of the cytoplasmic membrane caused by SMAP29 at low concentrations indicates that cytoplasmic membrane disruption might be involved in SMAP29's mechanism of action.

In contrast, OaBac5mini and OaBac7.5mini caused relatively low cytoplasmic membrane depolarisation compared to gramicidin S. OaBac5mini caused very little depolarisation at its MIC of 0.125μg/mL and had a maximum depolarisation of only 50% of that of gramicidin S.

OaBac7.5mini caused similar depolarisation at its MIC of 1.75μg/mL compared to SMAP29 at its MIC; however its maximum depolarisation was only 40% of that of gramicidin S. These results suggest that membrane depolarisation is not the mechanism of action used by these proline/arginine-rich peptides. Instead it was likely that this depolarisation was an intermediate step in the process. The peptides probably passed through the cytoplasmic membrane to interact with inner cellular contents. This mechanism of action has been displayed by PR-39, a porcine proline/arginine-rich peptide, which kills bacteria by stopping protein and DNA synthesis (Boman *et al*, 1993).

5.7 KILL CURVES

To further compare the way the peptides interact with Gram-negative bacteria, the optical density and viable cell count of *E. coli* O111 treated with the test peptides was monitored over time. The method used is described in Section 3.2.7. The log-phase bacteria cells were diluted to concentrations of approximately 2 x 10^7 CFU/mL in MHB and were incubated in a water bath at 37°C. The peptides were added to the cultures at twice their MIC concentrations. This experiment was carried only out once because of the large amount of each peptide required and the limited amounts available. The effect of the peptides on the optical density and viable cell count of *E. coli* O111 over time are shown in Figure 5.10.

The addition of SMAP29 to log-phase *E. coli* O111 reduced the number of bacterial cells by six-log in the first five minutes. After ten minutes no viable cells remained. The positive control, polymyxin B, also caused a rapid decrease in cell numbers, although not as quickly as SMAP29. Both of these peptides also caused the optical density to decrease slightly over time, which indicates that some cell lysis occurred. However, direct cell lysis was probably not the mechanism of action of SMAP29 because the drop in optical density was not as pronounced as it would be if this was the case (Zabucchi *et al*, 1983; Yourassowsky *et al*, 1985). This is consistent with the hypothesis that the higher membrane depolarisation caused by SMAP29, compared to the proline/arginine-rich peptides, leads to loss of the proton gradient. This results in leakage of essential molecules and cell death, but not necessarily complete cell lysis.

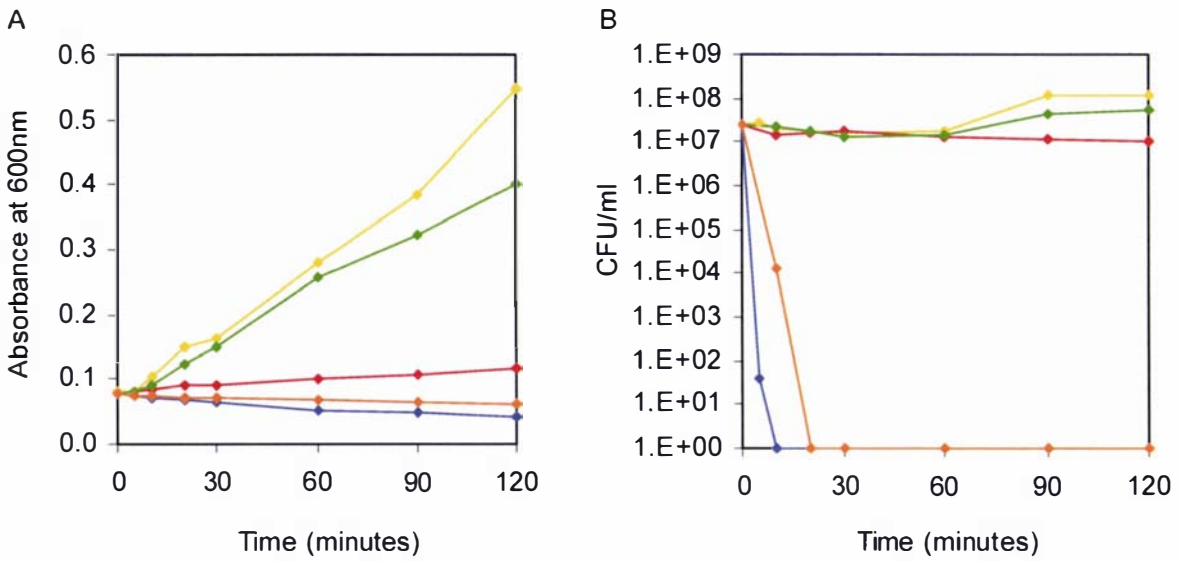

Figure 5.10 - Optical density and viable cell count over time for *E. coli* O111 treated with synthetic ovine peptides. Graph (A) shows the optical density and (B) shows the viable cell count. The samples used were *E. coli* untreated (—) and treated with 4μg/mL SMAP29 (—), 4μg/mL OaBac5mini (—), 32μg/mL OaBac7.5mini (—) and 4μg/mL polymyxin B (—).

In contrast, OaBac5mini and OaBac7.5mini did not induce the death of the bacterial cells. However, the addition of OaBac5mini resulted in no change to the optical density and viable cell counts, and this would suggest that this peptide stopped cell division. The cells treated with OaBac7.5mini had less of an increase in optical density and viable cell count compared to the untreated cells. This means that this peptide may have partially inhibited the division of the cells. Therefore, these two proline/arginine-rich peptides were not bactericidal, as was SMAP29, but they were bacteriostatic. This is consistent with the theory that they pass through the cytoplasmic membrane and interact with the inner cellular contents.

5.8 DNA BINDING

To determine whether the test peptides were able to bind to DNA, they were mixed with DNA at different ratios, and subjected to electrophoresis on an agarose gel (0.75%) according to the method described in Section 3.2.8. If the peptides bound to the DNA, they inhibited the migration of the DNA on the gel. Images of the gels are given in Figure 5.11. The lambda DNA used had been cut with the restriction enzyme Eco RI into fragments sized 21.2, 7.4, 5.8, 5.6, 4.8, and 3.5kbp. The two similarly sized bands, 5.6 and 5.8kbp, showed up as one wide band.

All three test-peptides inhibited the migration of the DNA. This was probably due to the peptides cross-linking the DNA to form larger molecules that could not migrate down the

agarose gel. Of the three peptides, OaBac5mini was the most effective at inhibiting the migration of the DNA. At a DNA:OaBac5mini ratio of 1:1, most of the DNA was bound, and at a ratio of 1:2 all the DNA was bound. OaBac7.5mini and SMAP29 required ratios of 1:4 to bind all of the DNA.

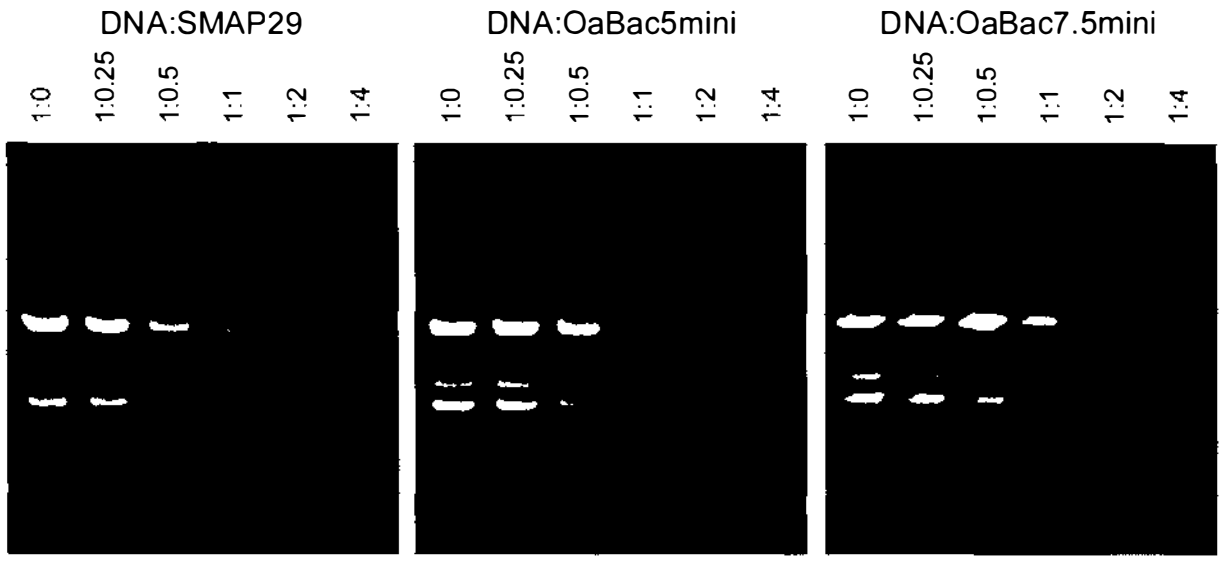

Figure 5.11 - DNA gel showing the running pattern of different ratios of DNA and synthetic ovine peptides.

These results indicate that the mechanism of action of the proline/arginine-rich peptides may involve interaction with bacterial DNA. OaBac5mini had a higher affinity for DNA than OaBac7.5mini, which may account for its lower MIC values shown in Section 5.2. OaBac5mini also had a higher affinity for DNA compared to SMAP29, even though SMAP29 is more cationic than OaBac5mini. This may be because the structure of OaBac5mini allows it to interact more readily with the DNA.

These results show that as well as depolarizing the cytoplasmic membrane of bacterial cells, SMAP29 can also interact with DNA. Previous studies have shown conflicting results for the mechanism used by SMAP29. Scanning electron microscopy images of *E. coli* and *S. aureus* cells treated with SMAP29 showed cells with numerous blebs and cell debris, which is consistent with the membrane interaction model (Skerlavaj *et al*, 1999). Similarly, confocal fluorescence microscopy showed that SMAP29 accumulated on the plasma membrane of fungal cells, which also suggests this is the target of the peptide (Lee *et al*, 2002). In contrast, immuno-electron microscopy was used to show that SMAP29 rapidly penetrated both the outer and inner membranes of *P. aeruginosa* and accumulated in the bacterial cytoplasm

(Kalfa *et al*, 2001). It implies that the mechanism of action used by SMAP29 changes depending on the organism and/or the conditions.

5.9 CONCLUSIONS

This first objective of the work presented in this chapter was to determine the spectra of activity of the three test peptides. The MIC tests confirmed that SMAP29 has potent antimicrobial activity against Gram-negative and Gram-positive bacteria and yeast. The proline/arginine-rich peptides were less active than SMAP29. OaBac5mini displayed potent activity, similar to SMAP29, against the Gram-negative bacteria, but it had only moderate activity against the Gram-positive bacteria and yeast. OaBac7.5mini was only moderately active against all the organisms tested, except the supersensitive mutants. However, both the ovine-derived Bac peptides were more active than their bovine analogs, Bac5 and Bac7 (Gennaro *et al*, 1989).

The second objective of the work presented in this chapter was to investigate the secondary structures of the peptides under different conditions. The circular dichroism studies showed that all three peptides had random structures in aqueous solutions, but in membrane-like conditions they formed amphipathic structures. In these conditions SMAP29 was α-helical as expected, and the proline/arginine-rich peptides, like their bovine counterparts, probably adopted a polyproline type II extended helix structure.

The final objective of the work presented in this chapter was to investigate the basis of the synthesised peptides' antimicrobial activities by assessing the way they interact with bacteria. The Gram-negative bacteria mechanism of action studies showed that all three peptides were able to bind to LPS, increase the permeability of the outer membrane and pass across this membrane to interact with the cytoplasmic membrane. SMAP29 caused substantial depolarisation of the cytoplasmic membrane, which indicates that the disruption of this membrane is involved in its mechanism of action. This was confirmed by the kill curve results, which showed that SMAP29 caused a large drop in viable cells and a slight decrease in optical density, which demonstrated that cell death had occurred. In contrast, OaBac5mini and OaBac7.5mini caused less cytoplasmic depolarisation and did not cause a drop in the viable cell count. Instead it was thought that these peptides passed across the cytoplasmic membrane and interacted with the inner cellular contents. It was shown that these peptides

were able to bind to DNA, so this may be their target. However, SMAP29 was also able to bind to DNA, which means it may have more than one mechanism of action.

These experiments indicate that SMAP29 has a different mechanism of action to OaBac5mini and OaBac7.5mini. This means that the innate immune system of sheep contains peptides that use at least two different mechanisms of action (inner membrane depolarisation and interaction with cytoplasmic contents) to fight an infection. The use of peptides with different mechanisms may be advantageous to the host animal because if an invading organism is resistant to one mechanism it may still be susceptible to the other, thus providing the animal with a backup mechanism for dealing with invading organisms. The use of peptides with different mechanisms would also make it more difficult for the microorganisms to build up resistance. This also means 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 utilising different mechanisms should be present, providing "insurance" for the product's effectiveness against a wide range of pathogens.

CHAPTER 6
MORPHOLOGY OF BACTERIAL CELLS TREATED WITH SYNTHETIC OVINE CATHELICIDINS

6.1 INTRODUCTION

The work presented in this chapter was concerned with the third objective of this research project, which was to investigate the morphological changes to microbial cells induced by ovine antimicrobial peptides. The previous chapter indicated that the proline/arginine peptides, OaBac5mini and OaBac7.5mini, had a different mechanism of action to that of α-helical SMAP29. The aim of the research presented in this chapter was to investigate whether the test peptides induced changes in the bacterial cell morphology and whether these changes confirmed the mechanisms of action proposed in the previous chapter (Chapter 5).

The results presented in Chapter 5 indicated that SMAP29 caused rapid cell death by depolarising the cytoplasmic membrane, which resulted in leakage of the cellular contents, and some complete cell lysis. In contrast, OaBac5mini and OaBac7.5mini appeared to inhibit cell division by interacting with the inner cellular contents. It was hypothesised, therefore, that SMAP29 would cause the morphology of the cells to change considerably, whereas the proline/arginine-rich peptides would cause little or no change. Of the two proline/arginine-rich peptides, only OaBac5mini was used for these tests because OaBac5mini and OaBac7.5mini appeared to act on cells in a similar manner. OaBac5mini was chosen because it was the more active of the two peptides.

To get a complete view of the morphology of the peptide-treated cells two techniques, transmission electron microscopy (TEM) and atomic force microscopy (AFM), were used. As discussed in Section 2.7.1, TEM gives an electron density image of cell cross-sections; whereas, AFM gives topological images of the cell surface. Together these techniques should produce a good overall model of the cell morphology, which can be used to confirm the proposed mechanisms of action of the respective peptides.

6.2 *E. COLI* TEM RESULTS

E. coli O111 cells were untreated, or treated with SMAP29 or OaBac5mini for one hour. The method used is described in Section 3.2.1. The samples were fixed and cut into thin sections.

For each treatment duplicate samples were prepared and numerous sections (approximately ten) were cut from each. The sections were examined using TEM and multiple images were taken of each section. The electron density images given in Figure 6.1 show typical results.

The images of the samples treated with SMAP29 were different to those of the untreated cells. The untreated cells were all uniformly shaped, with intact cell walls; whereas many of the SMAP29-treated cells had their cell walls missing. In Figure 6.1 the green arrows point to cells without cell walls, called ghost cells, and the yellow arrows point to cell walls that had separated from the cells. The images show that in many cases the cytoplasmic contents were leaking out of the cells. This is consistent with the results of the previous experiments in Chapter 5 that showed that SMAP29 disrupted the membranes of Gram-negative bacteria.

The images of the samples treated with OaBac5mini were similar to those of the untreated cells. However, the OaBac5mini-treated cells often had areas where the cell wall was not attached to the cytoplasmic membrane as shown by the red arrows in Figure 6.1. This is a typical sign that outer membrane permeabilisation has occurred. None of the OaBac5mini-treated cells appeared to be leaking their inner cellular contents, so inner membrane disruption had not occurred. This is further evidence that OaBac5mini has a different mechanism of action to SMAP29.

6.3 *S. AUREUS* TEM RESULTS

As with the *E. coli* cells, *S. aureus* 4163 NCTC cells were also untreated, or treated with SMAP29 or OaBac5mini for one hour. After treatment the samples were fixed, cut into sections and examined using TEM as described in Section 3.3.1. The electron density images given in Figure 6.2 show typical results.

The SMAP29-treated *S. aureus* cells were notably different to the untreated cells. The visible differences were similar to those seen in the SMAP29-treated *E. coli* culture. There were numerous ghost cells without cell walls, which are shown by the green arrows. Some intact parts of cell walls were also visible as shown by the yellow arrows. These results indicated that SMAP29 acted on Gram-positive cells similarly to the way it acted on Gram-negative cells.

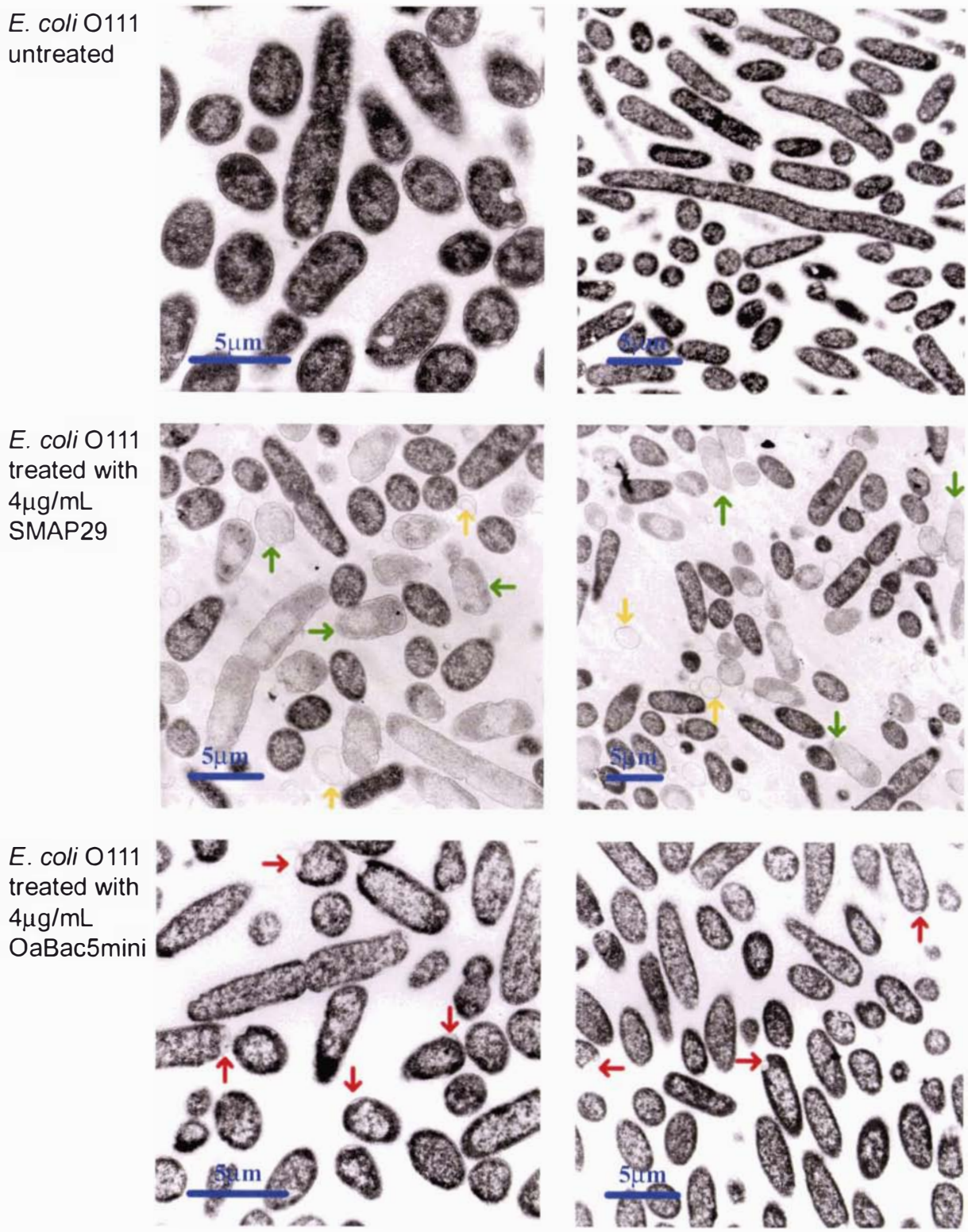

Figure 6.1 - Transmission electron microscope images taken of *E. coli* O111 cells treated with SMAP29 and OaBac5mini for one hour. The yellow arrows point to cell walls that have separated from the cytoplasm, the green arrows point to ghost cells that are not surrounded by a cell wall, and the red arrows point to cells where the cell wall is partially separated from the rest of the cell.

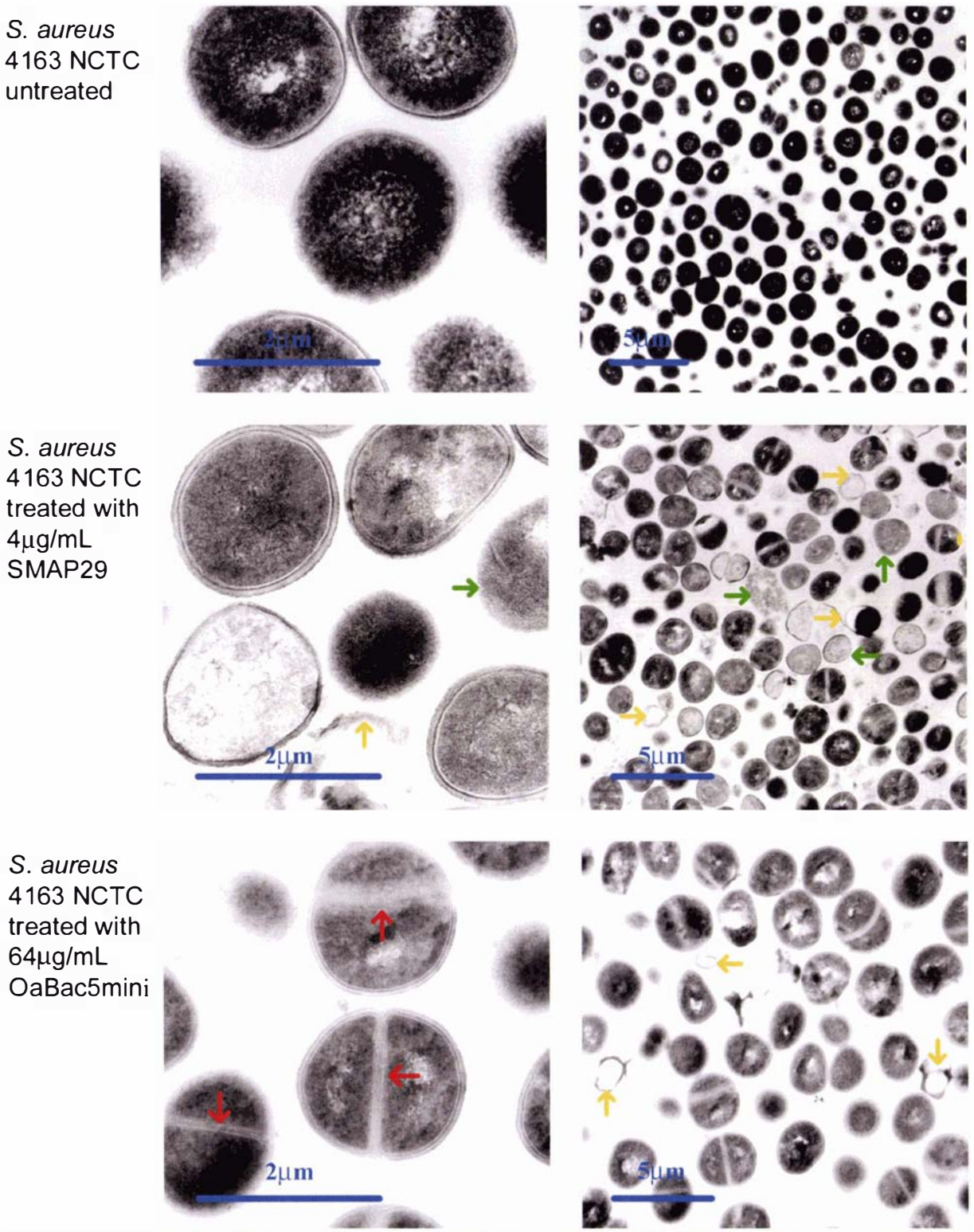

Figure 6.2 - Transmission electron microscope images taken of *S. aureus* 4163 NCTC cells treated with SMAP29 and OaBac5mini for one hour. The yellow arrows point to cell walls that have separated from the cytoplasm, the green arrows point to ghost cells that are not surrounded by a cell wall and the red arrows point to the septa in the dividing cells.

In contrast, the OaBac5mini-treated *S. aureus* cells did not appear to be damaged as much as were the *E. coli* cells treated with this peptide. There were no areas where the cell wall had separated from the cytoplasmic membrane, but some cell wall debris was present as shown by the yellow arrows. Compared to the control cells, the OaBac5mini treated culture contained a large number of cells with septa, which are indicated by the red arrows. This may be because the peptide stopped the division of the cells at this point; whereas, the control cells continued to divide normally. This is consistent with the theory that the proline/arginine-rich peptides inhibit cell division as indicated in Chapter 5.

6.4 *S. AUREUS* AFM METHOD DEVELOPMENT

Before AFM could be used to image the surface of bacterial cells, a procedure for preparing the bacterial cells for imaging by AFM had to be developed, because the application of AFM to image cells is relatively new. As discussed in Section 2.7.1 of the literature review, AFM was an attractive method because, unlike other methods like SEM, it can be used to view both dry cells and also cells in a liquid medium. This latter feature was particularly appealing as it meant that images could be taken in real time to follow the changes occurring to the cell morphology when treated with the peptides. Numerous difficulties, notably immobilization of cells, were encountered when measuring changes to the morphology of dried bacteria, so the technique was never employed with wet samples. Three techniques for immobilising bacterial cells were investigated. The first looked at the use of filter membranes, the second the use of agar gel surfaces and the third the use of a glass slide.

The filter membrane method was tried first because this was the most common method reported in the literature for the immobilisation of cells for AFM imaging (Kasas and Ikai, 1995). Unfortunately this method did not work well for this application. The membranes used to immobilise the cells were polycarbonate membranes, which are very smooth. Images of *S. aureus* 4163 NCTC cells trapped on a polycarbonate membrane are shown in Figure 6.3. The left image is the height image. In this image the darker colours show areas that were lower and the lighter colours show areas that were higher. Therefore, the dark areas are the pores in the membrane. Three of the pores contained visible cells as shown by the arrows, so these areas are lighter. As an alternative way to display the same information, the right image shows the deflection. In this image the dark areas represent places where the height of the sample was decreasing and the light areas represent places where the height of the sample was increasing as it was scanned by the AFM tip from left to right.

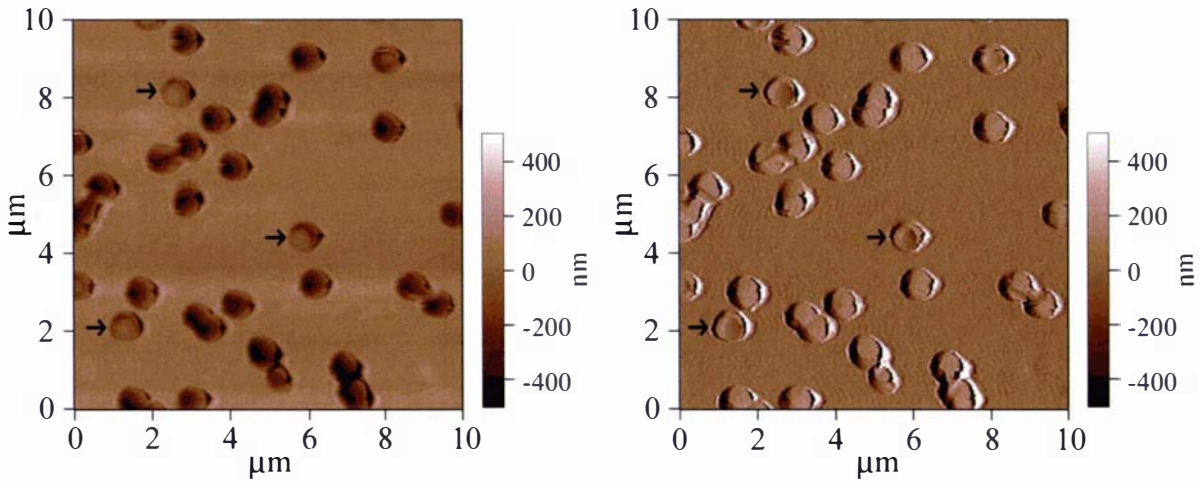

Figure 6.3 - AFM images of *S. aureus* NCTC 4163 cells trapped on a polycarbonate membrane. The image on the left is the height image and that on the right is the deflection image. The arrows point to membrane pores which contain cells.

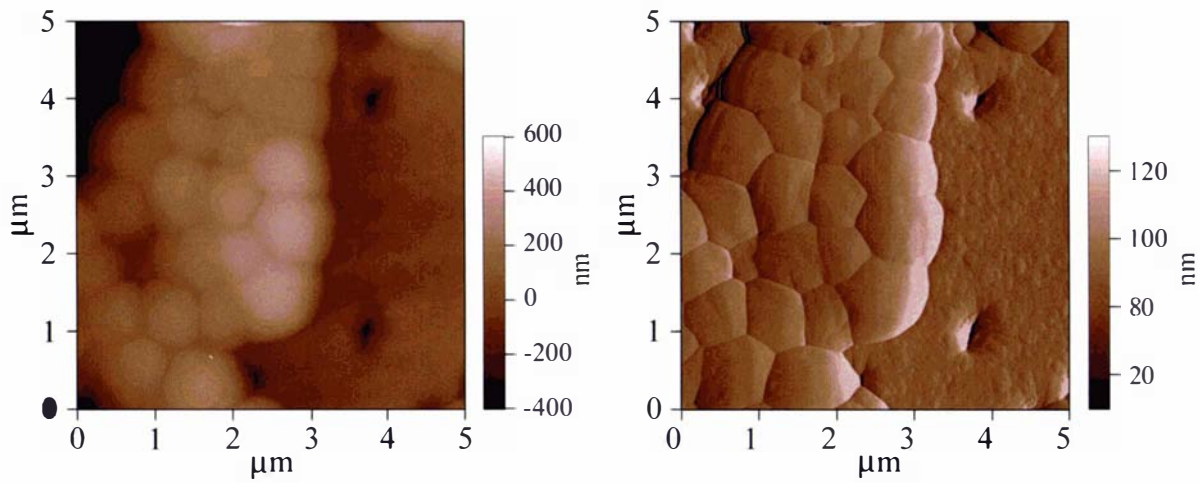

Figure 6.4 - AFM images of *S. aureus* NCTC 4163 cells grouped together on a polycarbonate membrane. The image on the left is the height image and that on the right is the deflection image.

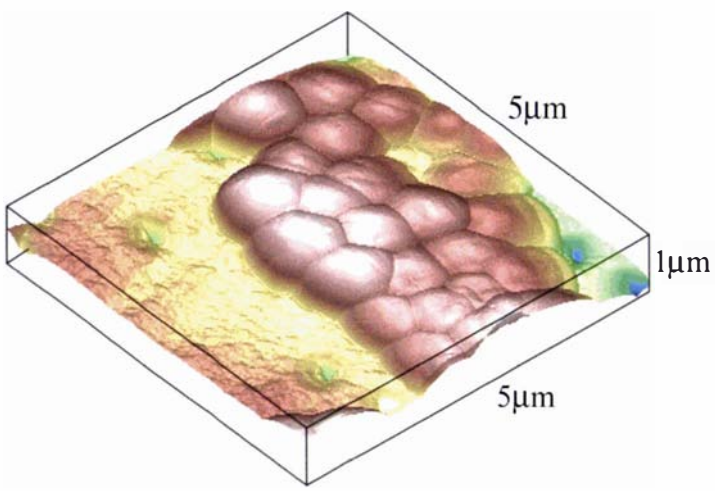

Figure 6.5 - AFM 3D representation of *S. aureus* NCTC 4163 cells grouped together on a polycarbonate membrane.

One problem with the use of the polycarbonate membranes was that the cells often formed bunches on top of the membrane instead of being entrapped in the pores as shown in Figure 6.4 and Figure 6.5. This made it difficult to get complete images because the cells were easily detached from the membrane during imaging.

Problems also arose with the antimicrobial treated cells. Few cells were present on the membranes if they had been treated with the antimicrobial peptides prior to membrane entrapment. This was probably because many of the cells were disrupted. The weakened cells and cell fragments may have passed through the pores in the membrane instead of being entrapped. This made it difficult to locate treated cells for imaging.

When the cells were treated with an antimicrobial peptide, there were also problems capturing images of the few cells that were trapped in the membrane because they often disappeared during imaging. For example, in a test carried out using commercially-produced nisin as the test peptide, initially two cells were trapped in the membrane pores but on the second pass only one of the cells was left. These images are shown in Figure 6.6. This was probably because the cells were weakened by the peptide, so they were easily pushed through the pores by the AFM tip.

As an alternative to the membrane entrapment method, another method, which immobilised the cells on a gel surface, was attempted (Goldman *et al*, 1997). However, this method was not successful because the AFM tip cut into the agar instead of scanning its surface. If this method is to be used in the future, more work is needed to determine the correct way to prepare the agar surface, and to immobilise the cells on top of the agar.

The final technique tested, which involved drying the cells on a glass slide (Braga and Ricci, 1998), was the most successful. For this method log-phase cells were collected by centrifugation, then washed and resuspended in water. A drop of the cell suspension was put onto a glass slide and allowed to dry at room temperature. This technique gave clear images of the cells against a smooth background.

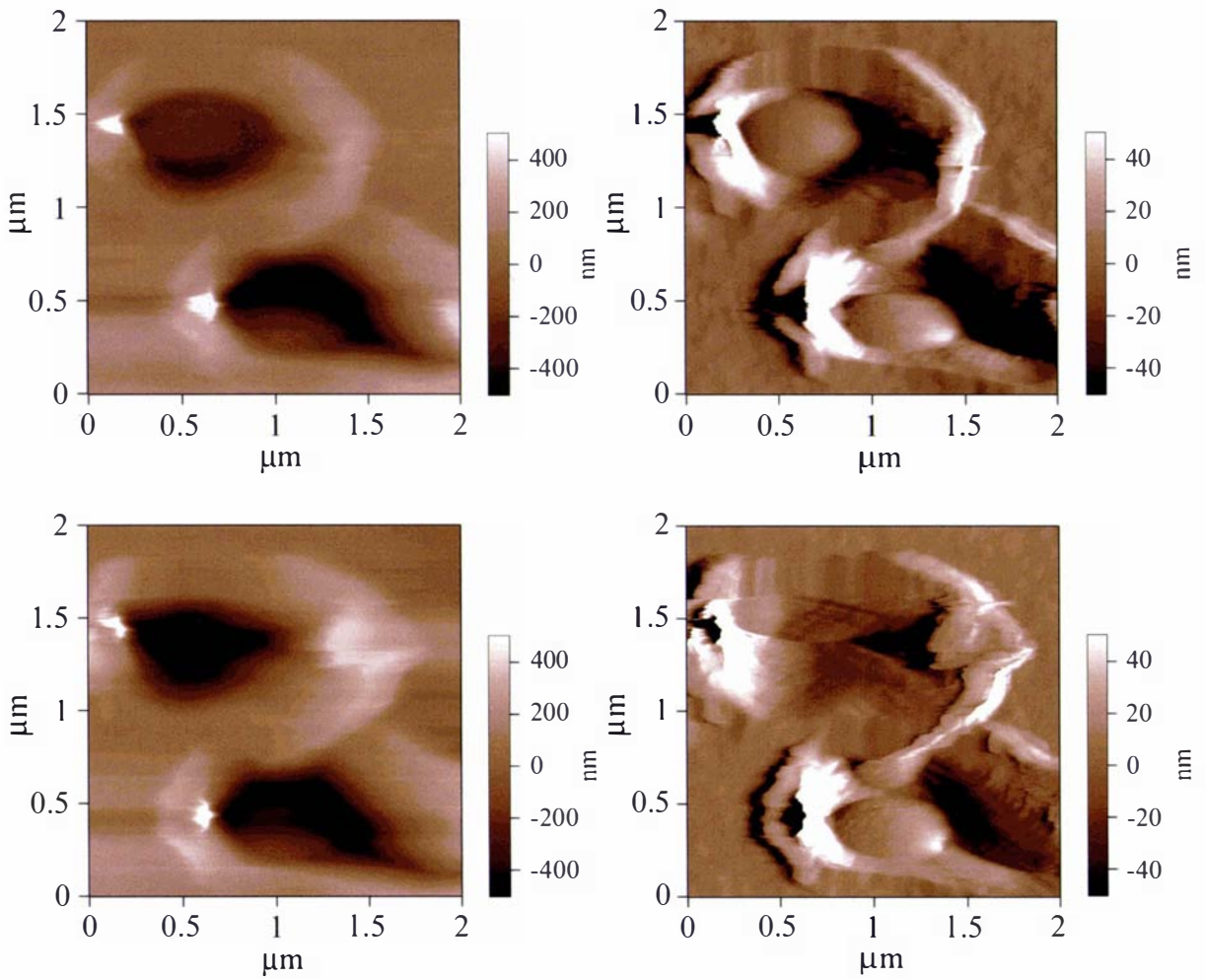

Figure 6.6 - AFM images of *S. aureus* NCTC 4163 treated with 25µg/mL nisin. Initially two cells were present (top images) but after a few minutes only one cell remained (bottom images). The images on the left are the height images and those on the right are the deflection images.

6.5 *S. AUREUS* AFM RESULTS

The glass slide technique described above was used to compare the surface topology of untreated *S. aureus* NCTC 4163 cells with those that were treated with SMAP29 or OaBac5mini. The details of the method are given in Section 3.3.2. For each treatment method two slides were prepared and numerous sections of each slide were chosen randomly for imaging. The results are shown as both an overview of numerous cells in Figure 6.7 (30µm x 30µm) and as a close up of one or two cells in Figure 6.8 (2µm x 2µm). These images are typical of those collected. The apparent sloping of the left hand side of the cells in Figure 6.8 is an artefact of the method due to contact with the side rather than the point of the tip.

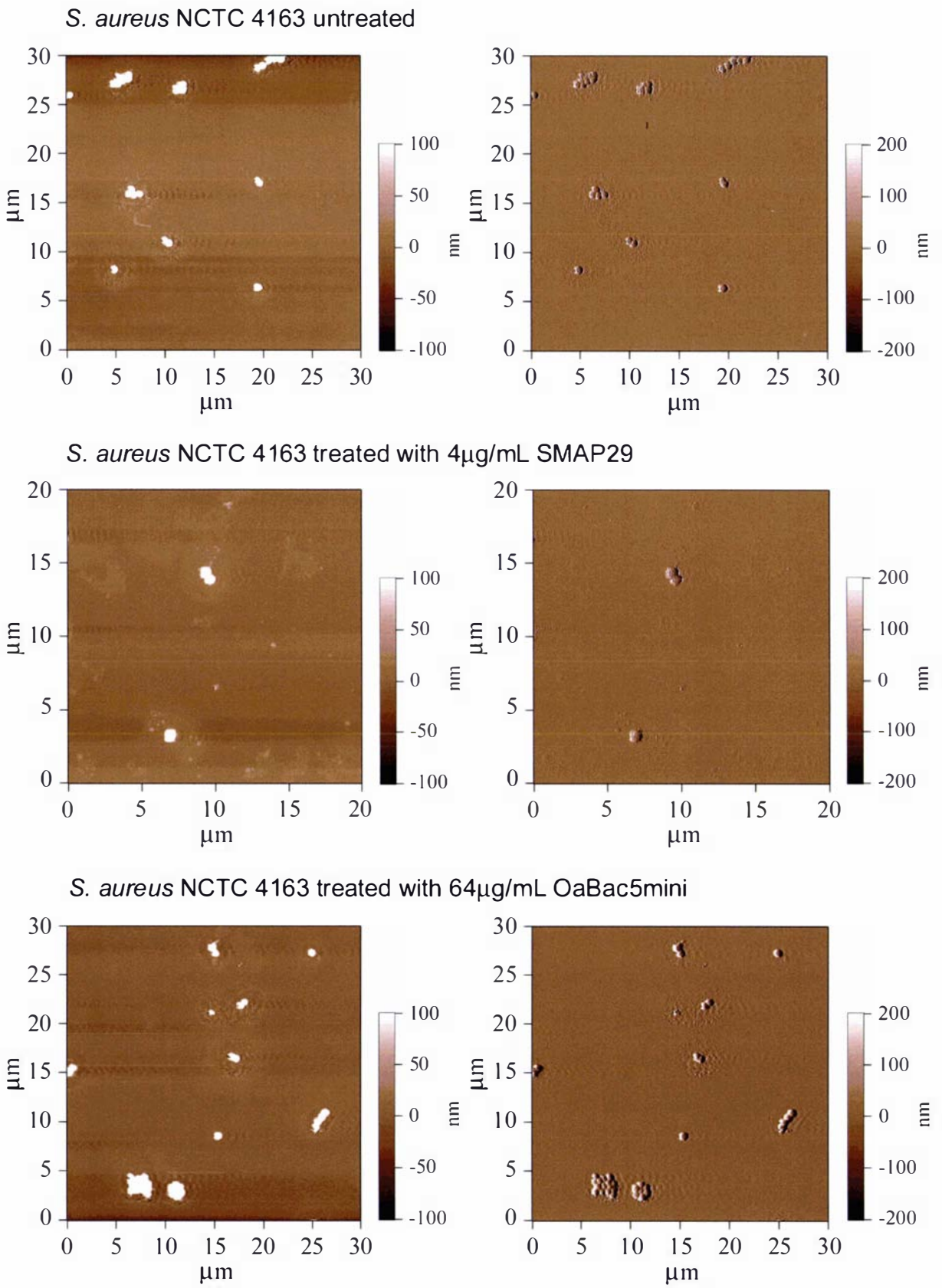

Figure 6.7 - Far away AFM images of *S. aureus* NCTC 4163 cells on a glass slide treated with SMAP29 and OaBac5mini for 30 minutes. The images on the left are the height images and those on the right are the deflection images.

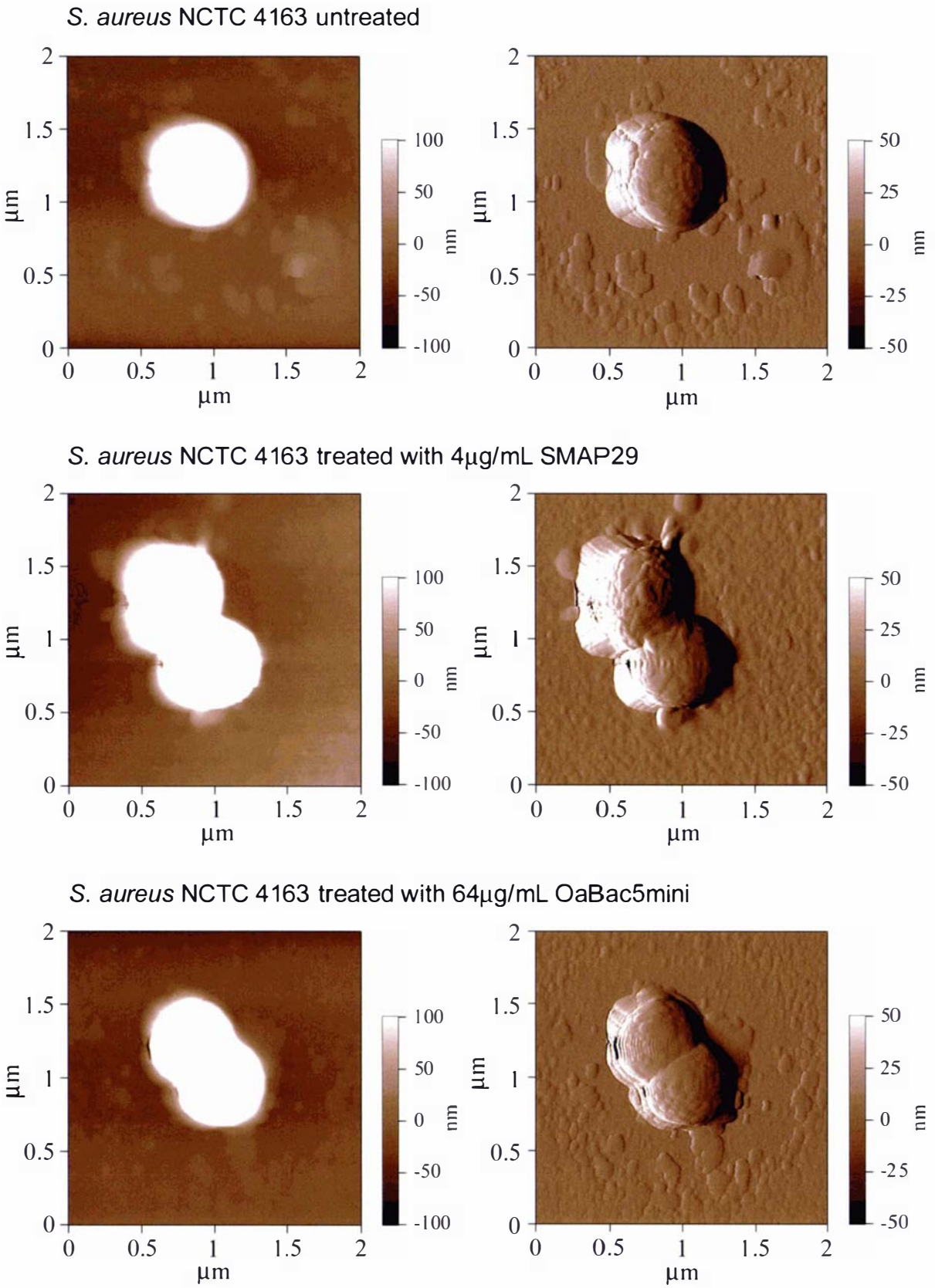

Figure 6.8 - Close up AFM images of *S. aureus* NCTC 4163 cells on a glass slide treated with SMAP29 and OaBac5mini for 30 minutes. The images on the left are the height images and those on the right are the deflection images.

The AFM images of the untreated cells showed that the slides were covered with a thin layer of unknown material. This material could be solids from the growth media; however, the cells were washed before drying. Directly around the bacterial cells were zones where this material was not present. This can be seen in Figure 6.8. The material on the edge of the clear zones was different to that which covered the rest of the slide. It was in clumps that were higher than the thin film covering. These rings of built-up material around the cells can be seen in Figure 6.7. It appears that the cells repelled the media solids during the drying process but the reason why this occurred is not known.

The AFM images of the cells treated with OaBac5mini were similar to those of the untreated cells. The clearings directly around the cells can be seen in Figure 6.8 and the clumpy material in rings were both present in Figure 6.7. This is consistent with the idea that the mechanism of action of OaBac5mini does not disrupt the membranes of the bacterial cells and cause the cellular contents to leak out, thus supporting the results described in Chapter 5

In contrast, the AFM images of the cells treated with SMAP29 were different to those of the cells that were untreated or treated with OaBac5mini. When areas were randomly selected for imaging, for the slides of the untreated and the OaBac5mini-treated samples, each image contained numerous cells; whereas for the SMAP29-treated samples, each image only contained a few cells, as seen in Figure 6.7, and sometimes no cells. SMAP29 may have weakened the cell walls and caused the cells to be leaky. This may have made them prone to lysis during the drying process.

Unlike the other samples, which only had built up material in rings around the cells, the SMAP29-treated sample contained a number of piles of material. This can be seen in Figure 6.7 and Figure 6.8. This material was probably cell debris from lysis of the cells induced by the peptide and the drying process. This is consistent with the proposed mechanism of action for SMAP29 in Chapter 5, which indicates that this peptide caused disruption of the cell membranes.

For the SMAP29-treated samples, the unknown material coating the slides came right up to the edges of the cells that were still intact; whereas, there were clear rings around cells treated with OaBac5mini and also with the controls (Figure 6.8). This implies that the SMAP29-treated cells were different to the untreated and OaBac5mini-treated cells; however, the reasons for this are not known.

6.6 *E. COLI* AFM METHOD DEVELOPMENT PROBLEMS

A considerable amount of time was spent trying to get good AFM images of *E. coli* cells; however, the AFM imaging of *E. coli* was not successful due to the nature of this bacterium. The method used to image *S. aureus* cells on a glass slide required the cells to be washed and resuspended in distilled water, but the *E. coli* cells were unable to withstand the osmotic pressure when suspended in water. *E. coli* is a Gram-negative bacterium so its cell wall is weaker than that of the Gram-positive *S. aureus* cells because of the difference in the thickness of the peptidoglycan layer. This resulted in the *E. coli* cells bursting as shown in Figure 6.9.

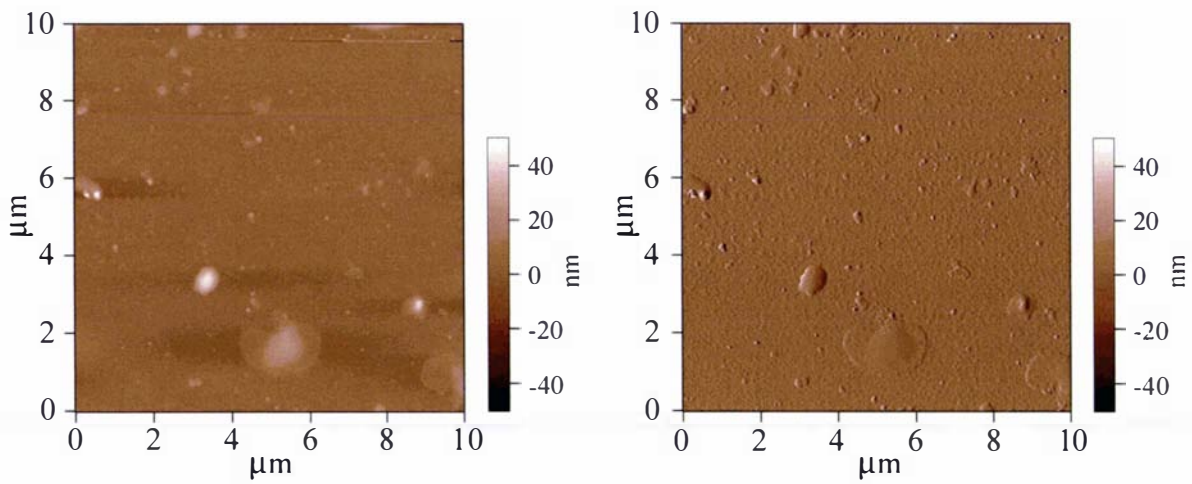

Figure 6.9 - AFM images of *E. coli* O111 debris after being suspended in distilled water. The images on the left are the height images and those on the right are the deflection images.

To try to overcome this lysis problem, the *E. coli* culture was resuspended in MHB instead of water. This was not successful because the cells were buried under a layer of dried media solids as shown in Figure 6.10. From these images it is apparent that the *E. coli* cells had a much rougher texture than the *S. aureus* cells. This is also probably due to the differences in the cell wall composition of the two bacteria.

As another alternative to attempt to image the *E. coli* cells, the culture was resuspended in phosphate buffer. The *E. coli* cells were stable in this solution because the salts reduced the osmotic pressure and stopped cell lysis. It was hoped that the cells would be more visible, than they were in MHB, because the phosphate buffer did not contain numerous large molecules as did the medium. However, when dried, this buffer formed crystals on the slide and covered the cells as shown in Figure 6.11.

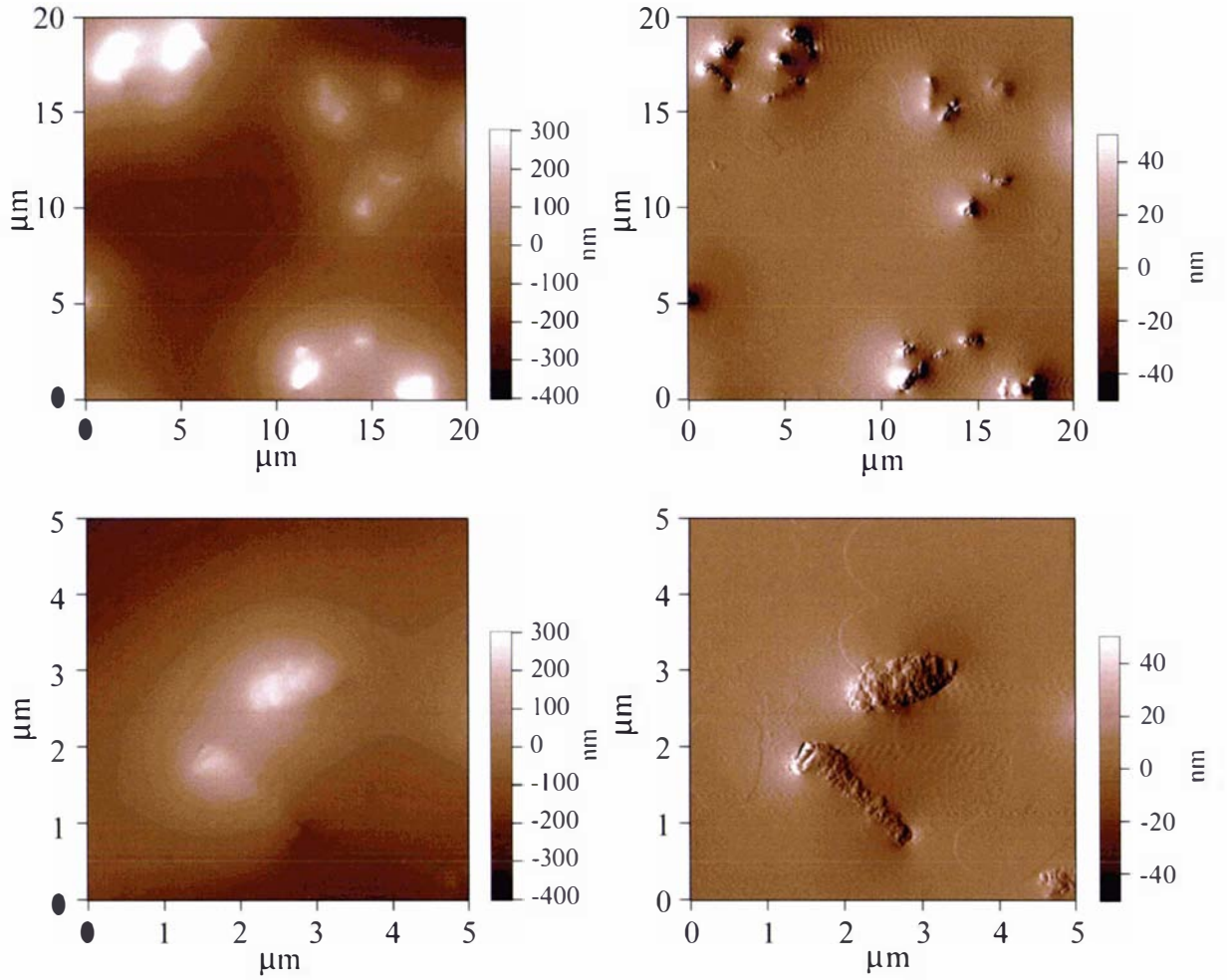

Figure 6.10 - AFM images of *E. coli* O111 cells covered with dried Mueller-Hinton broth. The images on the left are the height images and those on the right are the deflection images.

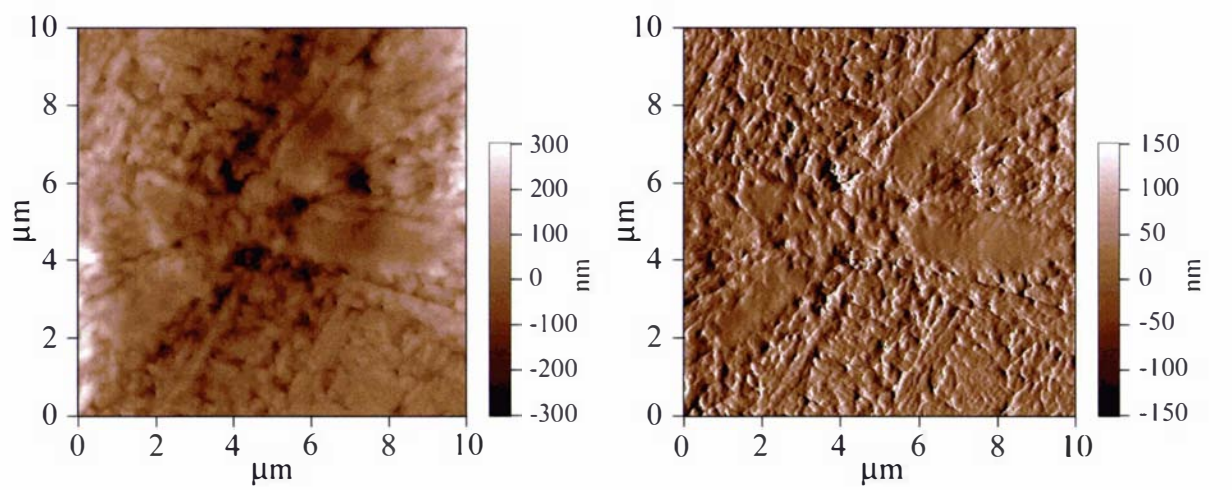

Figure 6.11 - AFM images of crystals that formed when *E. coli* O111 was suspended in phosphate buffer and dried on a glass slide. The images on the left are the height images and those on the right are the deflection images.

To successfully image the Gram-negative bacteria for the purposes of examining their cell morphology a different method to the glass slide method is required. One possibility is the membrane filter method because the media would pass through the pores in the membrane filter. However, *E. coli* cells are rod shaped so they would not easily be entrapped in the round pores. Another possibility is the gel entrapment method. This is probably the most suitable method because the cells would be stable in the agar, but more work is required to develop this technique.

6.7 CONCLUSIONS

The objective of the work presented in this chapter was to investigate the morphological changes to microbial cells induced by ovine antimicrobial peptides. As predicted, the two microscopy techniques showed that SMAP29 caused significant morphological changes in *E. coli* and *S. aureus* cells. In the TEM images of both *E. coli* and *S. aureus* cells treated with SMAP29, a large number of cells were missing their cell walls and some of these appeared to be leaking out their cytoplasmic contents. In the AFM images of *S. aureus* cells treated with SMAP29, there were a lot fewer intact cells than for the control and there was material present that was probably cell debris. These results support the earlier proposed theory that SMAP29 disrupted cell membranes of Gram-negative bacteria, which then weakened the cells and led to leakage of the inner cellular contents (Chapter 5). These results showed that SMAP29 also acted on Gram-positive bacteria in the same was that it acted on Gram-negative bacteria.

In contrast, the two microscopy techniques showed that OaBac5mini caused only minor morphological changes to occur in the *E. coli* and *S. aureus* cells. In the TEM images of the *E. coli* culture, some of the cells had sections where the cell wall was separated from the rest of the cell, but otherwise the cells appeared to be intact , i.e., the partial loss of the membrane did not weaken the cells in any way and as a consequence no lysis occurred. In the TEM images of the *S. aureus* culture, there were a lot more cells with septa compared to the control. This may have been because OaBac5mini stopped cell division as predicted from the mechanism of action study in Chapter 5. The AFM images of the *S. aureus* culture showed no obvious differences between the OaBac5mini-treated cells and the untreated control cells. These results confirm, as determined earlier, that unlike SMAP29, OaBac5mini does not disrupt the cell membrane and induce cell lysis.

TEM provided informative images of the cross-section of bacterial cells; however, AFM did not give as much information about the surface of bacterial cells as was hoped. AFM was technically difficult so a lot of time was spent developing the method. Eventually, dry *S.* aureus cells were successfully imaged but *E. coli* cells were not. It was hoped that AFM could be used to image live cells in liquid so that the changes induced by the peptide could be imaged in real time; however, this was not possible. Instead of AFM, it may be better to use another technique, such as SEM for future work.

CHAPTER 7
FACTORS AFFECTING THE ANTIMICROBIAL ACTIVITY OF SYNTHETIC OVINE CATHELICIDINS AGAINST *E. COLI* O157:H7

7.1 INTRODUCTION

The work presented in this chapter was concerned with the fourth objective of this research project, which was to determine the effect of different environmental factors on the activity of ovine antimicrobial peptides. In the previous chapters the composition of the antimicrobial peptides isolated from ovine blood neutrophils and the mechanism of action of the synthetic ovine peptides were described. This work investigated the antimicrobial activity of the peptides in a variety of conditions, to determine what applications the peptides may be suitable for. In conjunction with the separation of the major plasma proteins (serum albumins, transferrins, antibodies etc), the antimicrobial peptides extracted from ovine blood have potential to be utilised as, or in, high-value products as discussed in Section 2.4.2. For example, a mixture of peptides could be applied as a topical cream for cuts and grazes, or used as a biopreservative for chilled lamb products.

The aim of the work reported in this chapter was to gather information about factors that may enhance or inhibit the activity of the test peptides. The bactericidal activities of these peptides were compared under various conditions, including the addition of salt or metal ions to the media, altering the pH of the media, and heating the peptides before testing. Each of the factors was examined independently to see whether they had a significant effect on the antimicrobial activity of the peptides. Studies were also carried out to determine if there was synergistic activity between the test peptides, or between the peptides and common antibiotics.

Due to the limited amount of synthesised peptides available, only one organism, *E. coli* O157:H7, was used for these tests. This Gram-negative bacterium was chosen because it is a dangerous food-pathogen that has been known to contaminate meat products (Garcia-Olmedo *et al*, 1998). Normal *E. coli* inhabits the intestines of all animals, where it suppresses the growth of harmful bacteria and synthesises vitamins. *E. coli* O157:H7 is a pathogenic variety of *E. coli* that produces large quantities of one or more potent toxins that cause severe damage

to the lining of the intestine. The symptoms of the illness include severe stomach cramps and watery or bloody diarrhoea. If the antimicrobial peptides are to be used as an effective meat biopreservative they would need to be active against this organism.

7.2 EFFECT OF SALT

The first factor that was investigated to see if it affected the activity of the test peptides was sodium chloride concentration. It is well documented that defensins are salt-sensitive (Evans and Harmon, 1995). However, some cathelicidins, including SMAP29, appear to be resistant to high salt concentrations (Travis *et al*, 2000).

The salt-sensitivity of the three test peptides was established by determining the MIC of each peptide against *E. coli* O157:H7 in a variety of NaCl concentrations from 0 to 250mM as described in Section 3.4.1. The results are graphed in Figure 7.1. The raw data are given in Appendix A3.1, example calculations are given in Appendix A3.5 and the statistical analysis is given in Appendix A3.6. The analysis of variance determined that there were significant differences between the mean MICs of each peptide (p-value = 0.0019), and that there were significant differences between the mean MICs at different salt concentrations (p-value = 0.0024).

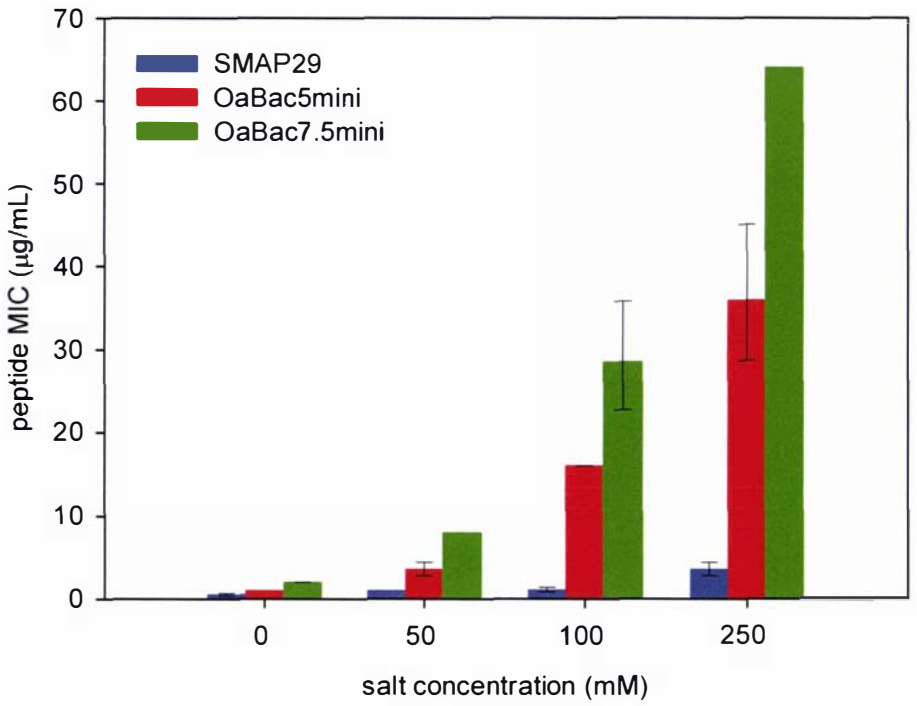

Figure 7.1 - The effect of salt concentration on the minimum inhibitory concentration (MIC) of synthetic ovine peptides against *E. coli* O157:H7. The bars show the geometric means of three runs of duplicate samples and the error bars show the 95% confidence intervals for these means.

As expected the MICs for each peptide increased with increasing ionic strength. However, SMAP29 had only a two-fold increase in MIC from 0 to 100mM NaCl so its activity was relatively stable in saline conditions. This agrees with previous results, which showed that SMAP29 is active at high salt concentrations (Travis *et al*, 2000; Shin *et al*, 2001a; Shin *et al*, 2001b). OaBac5mini and OaBac7.5mini were affected more than SMAP29 by the increased ionic strength. Both peptides had MIC values 16-fold higher at 100mM NaCl, than in the absence of salt. Another study showed that a purified variant of OaBac5 had decreased activity in the presence of 100mM NaCl, which is consistent with these results (Shamova *et al*, 1999). The decrease in activity is probably due to either the Na^+ ions competively binding to the bacterial LPS or the Cl^- ions binding to the peptide. Both cases would decrease the binding of the peptide to the outer membrane of the bacteria.

If the peptides were to be effective preservatives for chilled lamb products they would need to be active at the salt concentrations present in the meat. The concentration of salt in average, trimmed, raw lamb meat is 2mmoles/100g (Chan *et al*, 1995). For comparison, the 100mM solution tested is equivalent to 10mmoles/100g (100mM = 100mmoles/L = 100mmoles/kg = 10mmoles/100g). This means that the peptides are unlikely to be inhibited by the salt present in the meat.

7.3 EFFECT OF METAL IONS

The second factor to be investigated for its effect on the antimicrobial activity of the test peptides was metal ion concentration. Other studies have shown that the activity of cationic antimicrobial peptides is decreased even at low divalent cation concentrations (Selsted *et al*, 1985; Turner *et al*, 1998). It is believed that the first step in the mechanism of action of cationic antimicrobial peptides against Gram-negative bacteria is the binding of the peptides to the divalent-cation-binding sites of the polyanionic surface LPS (Devine and Hancock, 2002). The presence of cations in the solution may limit this binding ability by saturating the LPS divalent-cation-binding sites, and thereby decreasing the antimicrobial activity of the peptides.

To determine whether the test peptides were affected by the presence of cations, metal ions in the form of their chloride salts were added to the media at concentrations of 1, 5 and 10mM, and the MICs of the peptides were determined. The experimental method is described in Section 3.4.2. The results are summarised in Figure 7.2. The raw data are given in Appendix

A3.2, example calculations are given in Appendix A3.5 and the statistical analysis is given in Appendix A3.6. The analysis of variance determined that there were significant differences between the mean MICs at different concentrations for each of the cations tested (p-values were 0.0022, 0.0022, 0.0002 and 0.0000 for Na^+, K^+, Mg^{2+} and Ca^{2+} respectively). There were significant differences between the mean MICs of each peptide in the presence of K^+ and Ca^{2+} (p-values were 0.0031 and 0.0268), but not in the presence of Na^+ and Mg^{2+} (p-values were 0.1042 and 0.0671).

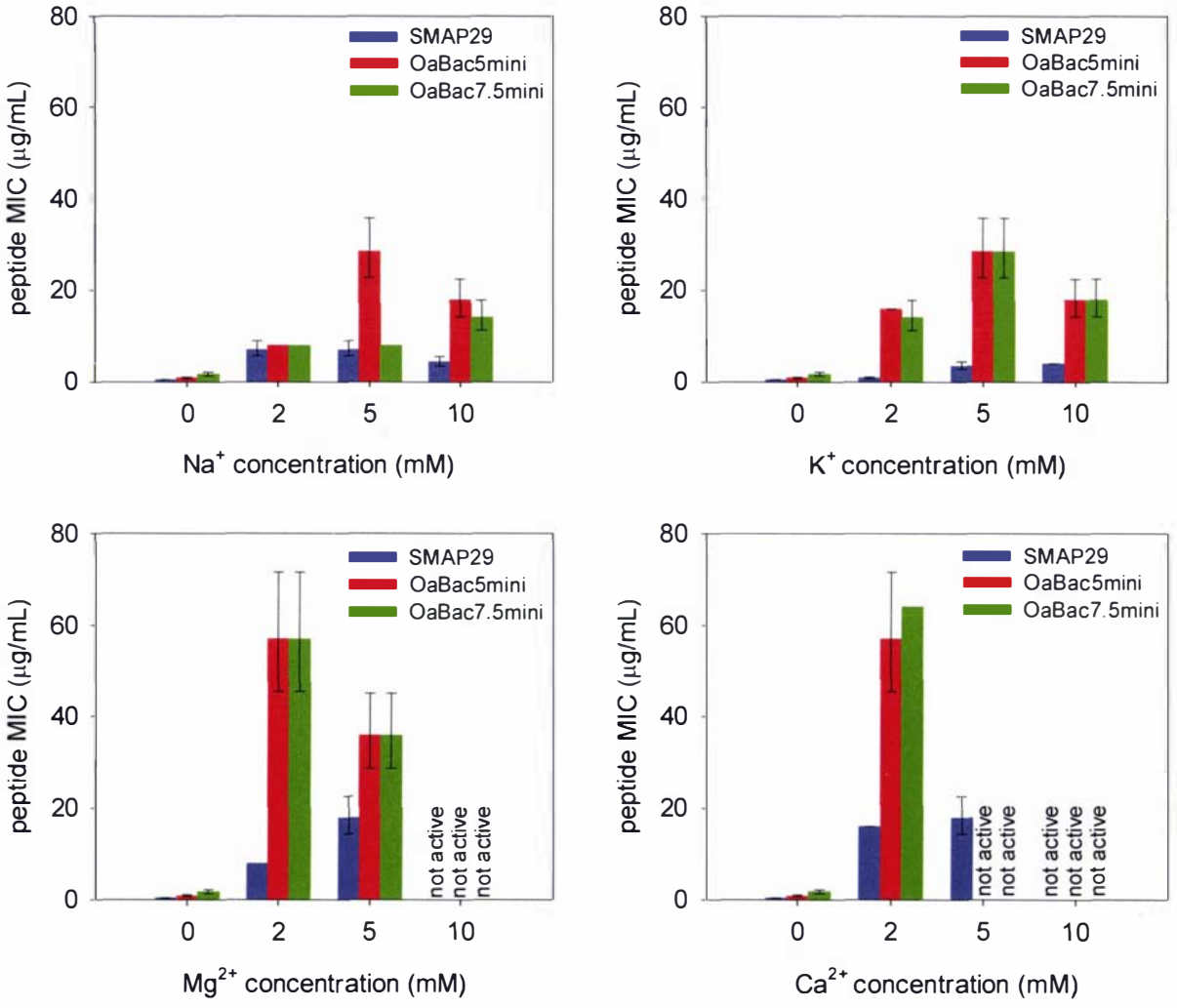

Figure 7.2 - The effect of metal ion concentrations on the minimum inhibitory concentration (MIC) of synthetic ovine peptides against *E. coli* O157:H7. The bars show the geometric means of three runs of duplicate samples and the error bars show the 95% confidence intervals for these means.

In the presence of the monovalent cations, Na^+ and K^+, the MICs of the test peptides were higher than in their absence. However, the peptides still showed moderate antimicrobial activity under these conditions, which shows that the monovalent ions do not completely block the binding of the cationic peptides to LPS.

The divalent ions had a larger effect on the activity of the test peptides than the monovalent cations. Mg^{2+} and Ca^{2+} ions inactivated the proline/arginine-rich peptides at concentrations of 10mM and 5mM respectively. SMAP29 was inactive in the presence of 10mM of both divalent cations. SMAP29's higher tolerance to divalent cations, compared to the other two peptides, was probably due to its higher affinity for LPS as shown in Section 5.4.

It has previously been shown that, like OaBac5mini and OaBac7.5mini, other cationic antimicrobial peptides are also more sensitive to Ca^{2+}, than Mg^{2+} ions (Turner *et al*, 1998) (Selsted *et al*, 1985). This is probably due to Ca^{2+} ions having a higher affinity for LPS than Mg^{2+} ions. This may be because Ca^{2+} ions are larger than Mg^{2+} ions and are able to interact more readily with the divalent-cation-binding sites on LPS.

For the proline/arginine-rich peptides there were some unexpected results. The mean MIC was significantly higher for OaBac5mini in the presence of 5mM compared to 10mM Na^+ (p-value = 0.0090). The mean MICs of both OaBac5mini and OaBac7.5mini were significantly higher in the presence of 5mM compared to 10mM K^+, and in the presence of 2mM compared to 5mM Mg^{2+} (p-value = 0.0090 in both cases). This implies that certain cation concentrations are more inhibitory than others; however, the reason for this is unknown.

Since it is envisaged that the antimicrobial peptides could be used as biopreservatives for chilled lamb products, the concentrations of the cations in fresh lamb meat were sought. The concentrations of Na^+, K^+, Mg^{2+} and Ca^{2+} in fresh, lean, raw lamb meat are 70, 330, 22 and 12mg/100g respectively (Chan *et al*, 1995). The concentrations of each cation in mmol/kg were calculated using Equation 7.1.

$$\text{mmol cation/kg raw lamb} = \frac{\text{mg cation/kg raw lamb}}{\text{g cation/mol}} \qquad \text{Equation 7.1}$$

e.g. For sodium:

$$\text{mmol sodium/kg raw lamb} = \frac{\text{mg sodium/kg raw lamb}}{\text{g sodium/mol}}$$

$$= \frac{70\text{mg sodium/kg raw lamb}}{22.99\text{g sodium/mol}}$$

$$= 3.04\text{mmol/kg raw lamb}$$

The calculated cation concentrations in raw lamb meat are summarized in Table 7.1. The divalent cations completely inhibited the antimicrobial activity at concentrations of 10mM,

which corresponds to 10mmoles/kg (10mM = 10mmoles/L = 10mmoles/kg), and were partially inhibitory at concentrations of 2mM, which corresponds to 2mmoles/kg. The concentrations of the individual divalent cations in the raw lamb meat are lower than the inhibitory concentrations; however, the inhibitory effect is most likely additive so the total divalent cation concentration should be considered.

Table 7.1 - Concentrations of metal ions in lean trimmed, raw lamb meat.

cation	mg/100g lamb	molecular weight	mmol/kg raw lamb
sodium	70	22.99	3.04
potassium	330	39.10	8.44
magnesium	22	24.30	0.91
calcium	12	40.08	0.30

The locations of the cations within the meat are also important when considering their possible interaction with the peptides and the bacterial LPS. Of the magnesium present in the meat, most of it is situated in the intracellular fluid at concentrations as high as 5-10mM (Price and Schweigert, 1987), so it is unlikely to interfere with the activity of the peptides on the surface of the meat. Similarly, in animals the majority of calcium is situated within the cells where is acts as an intracellular messenger (Price and Schweigert, 1987). However, in post-mortem meat free Ca^{2+} tends to rise and so could inhibit these peptides after the animal has died.

7.4 EFFECT OF PH

Another property that was important to investigate for its effect on the activity of the peptides was pH. This was established by determining the MIC of each peptide against *E. coli* O157:H7 at a variety of pH values from 5 to 9 according to the method described in Section 3.4.3. The results are graphed in Figure 7.3. There were significant differences between the mean MICs of each peptide (p-value = 0.0006), but there were no significant differences between the mean MICs at different pHs (p-value = 0.0851). The raw data are given in Appendix A3.3, example calculations are given in Appendix A3.5 and the statistical analysis is given in Appendix A3.6

Two of the test peptides had higher mean MICs in acidic conditions compared to basic conditions. OaBac5mini and OaBac7.5mini had MICs four to eight times greater at acidic pH than at pH8. The activity of these two peptides was inhibited more at pH 6 than pH 5. There was little change in the antimicrobial activity of SMAP29 over the pH range tested.

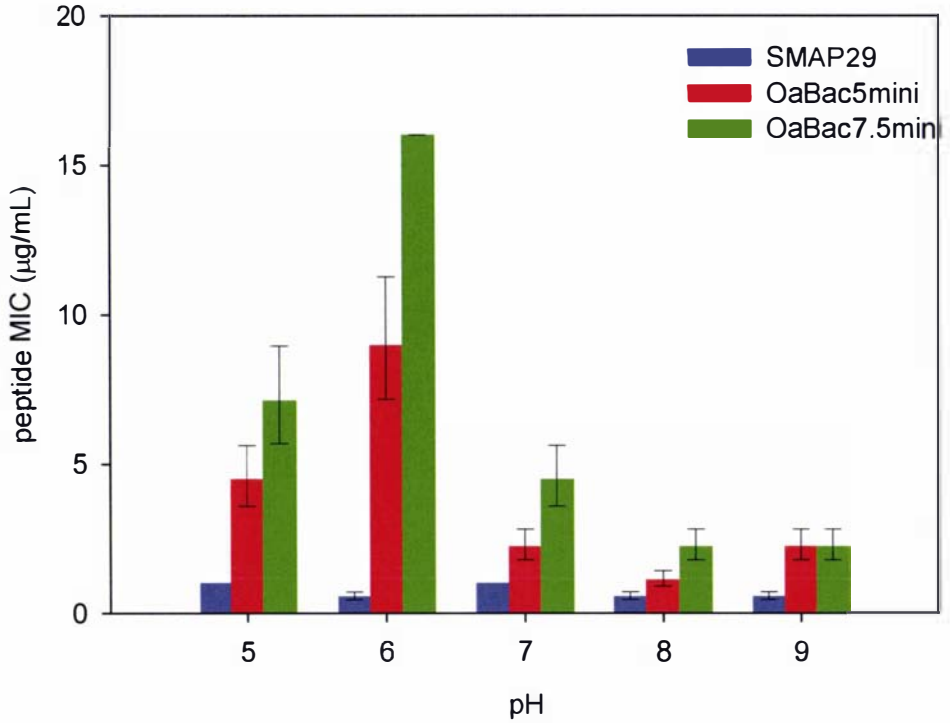

Figure 7.3 - The effect of media pH on the minimum inhibitory concentration (MIC) of synthetic ovine peptides against *E. coli* O157:H7. The bars show the geometric means of three runs of duplicate samples and the error bars show the 95% confidence intervals for these means.

The changes in activity at different pH values may be due to changes in the charges of the peptides. All three peptides contain numerous arginine residues; however, arginine has a pK of 12 (Stryer, 1995), so these residues would be positive in all the conditions tested. All three peptides also contain a terminal amino group, which has a pK of 8 (Stryer, 1995). It is unlikely that the protonation of this group causes the decrease in activity of the proline/arginine-rich peptides, because this also occurs in SMAP29, which is not affected. SMAP29 contains a histidine residue, which has a pK value of 6.5 (Stryer, 1995); however, the protonation of this residue does not appear to have an effect on the activity of the peptide. The peptides do not contain any other ionisable residues.

A decrease in antimicrobial activity at low pH has previously been shown to occur with other cationic antimicrobial peptides (Miyakawa *et al*, 1996). LL-37, a human cathelicidin, showed changes in its structure under different pH conditions (Johansson *et al*, 1998). At basic pH LL-37 is α-helical, but at acidic pH it has a random structure. Such conformational changes to the peptides may be responsible for their decreased activities in acidic pH conditions.

Alternatively, the changes in activity at acidic pH may be due to changes in the bacterial membranes, instead of the peptides. A reduction in the negative charge of the bacterial surface would decrease the binding of the cationic peptides.

If the peptides are to be used as a biopreservative they need to operate within the pH spectrum of meat products. The pH of the live animal is 7.2, but this falls to approximately 5.4 in the dead animal. The acceptable pH range for eating quality lamb is 5.3-5.7 (Oppenhei *et al*, 2003). This pH range is more acidic than the optimum for the proline/arginine-rich peptides; however, the peptides are still active in this range.

7.5 EFFECT OF TEMPERATURE

The final physical property that was investigated for its impact on the antimicrobial activity of the peptides was temperature. The effect of temperature on the activity of the peptides against *E. coli* O157:H7 was determined by heating the peptide solutions for 30 minutes prior to determining the MIC values as described in Section 3.4.4. The results are summarised in Figure 7.4. The raw data are given in Appendix A3.4, example calculations are given in Appendix A3.5 and the statistical analysis is given in Appendix A3.6. There were significant differences between the mean MICs of each peptide (p-value = 0.0000), and there were significant differences between the mean MICs at different temperatures (p-value = 0.0000).

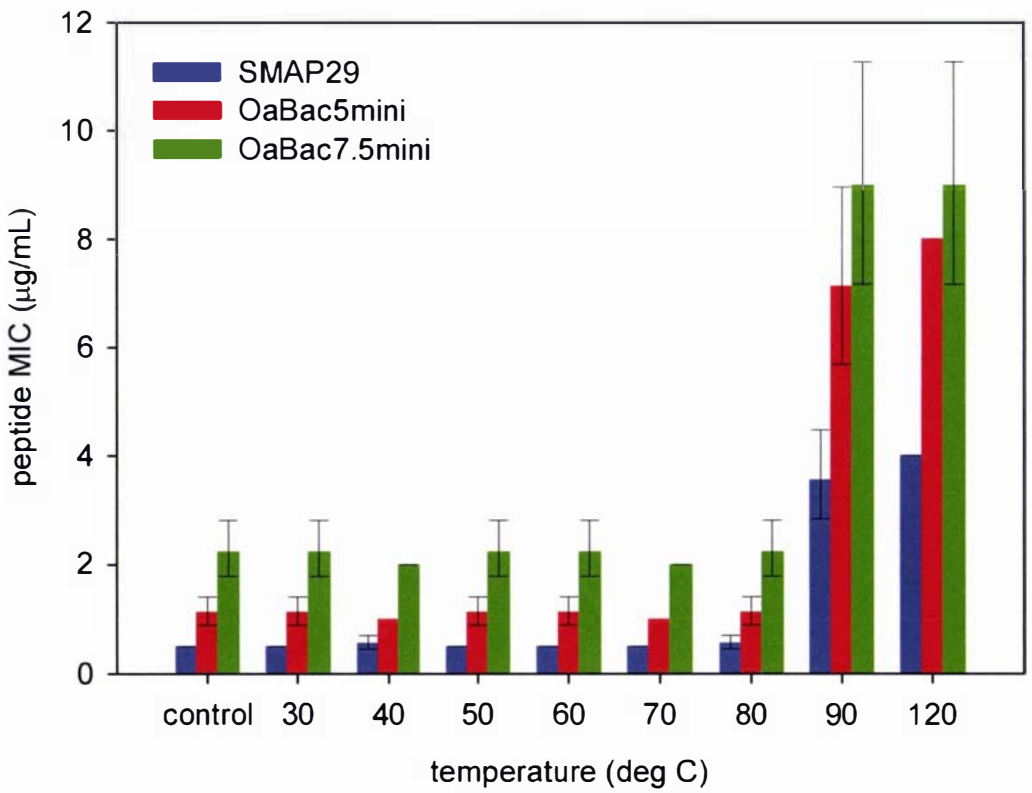

Figure 7.4 - The effect of heating on the minimum inhibitory concentration (MIC) of synthetic ovine peptides against *E. coli* O157:H7. The bars show the geometric means of three runs of duplicate samples and the error bars show the 95% confidence intervals for these means.

The peptides were relatively heat-stable. All three peptides retained their antimicrobial activity when heated to temperatures as high as 80°C. However, SMAP29 and OaBac5 had an eight-fold increase in their MICs after being heated to 90°C or 120°C, and OaBac7.5mini had a four-fold increase in MIC in these conditions.

It is common for heat-treatment to reduce the biological activity of peptides and proteins because it can cause them to unfold. The test peptides have simple secondary structures. In aqueous solutions the peptides have a random structure, and it is not until they come into contact with membrane-like conditions that they fold into their amphipathic structures, as shown by the CD spectra experiments in Section 5.2. The test peptides do not contain any disulphide bonds to stabilise them, which probably accounts for their decrease in activity after heating to high temperatures.

The reduction in activity of the peptides at high temperatures may limit their use. If they are to be used as preservatives for chilled lamb products, they would not be heated. However, they may not be suitable for other applications such as preservatives in canned goods, which are heated to high temperatures.

7.6 SYNERGY BETWEEN PEPTIDES

Along with exploring the effect of the environmental conditions on the activity of the peptides, their activity in combination with each other, compared to their individual activities, was investigated. Work carried out by other researchers has shown that some pairs of cathelicidins are synergistic, although not in all combinations (Yan and Hancock, 2001).

To determine if the test peptides worked synergistically together, the fractional inhibitory concentration (FIC) was calculated for each combination of the three peptides. Each pair of peptides was tested in a 96-well microtitre plate, with one peptide diluted down the columns and one across the rows. The details of the method are given in Section 3.4.5. The wells where growth occurred were recorded for each plate. A diagram of a typical FIC plate is given in Figure 7.5.

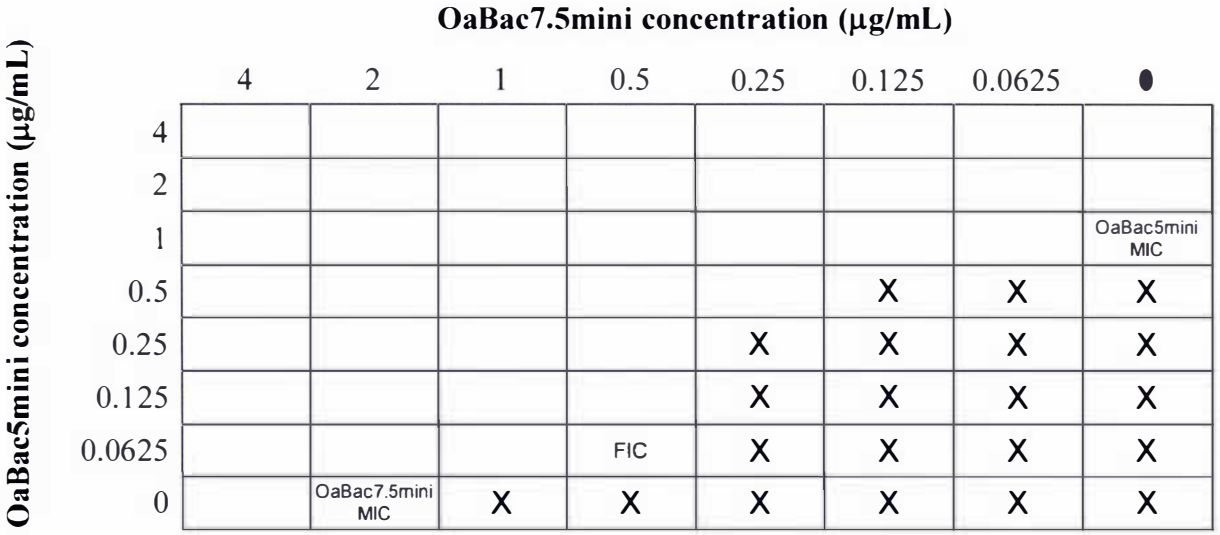

Figure 7.5 - Diagram of a microtitre plate for a typical synergy test for OaBac5mini and OaBac7.5mini. Growth occurred in the wells containing an 'X'. MIC = minimum inhibitory concentration. FIC = fractional inhibitory concentration.

The FICs were calculated using Equation 7.2.

$$FIC = \frac{[A]}{MIC_A} + \frac{[B]}{MIC_B} \qquad \text{Equation 7.2}$$

where MIC_A and MIC_B are the MICs of peptides A and B alone, and [A] and [B] are the minimum amounts of peptides A and B required for inhibition when used in combination.

e.g. For the typical OaBac5mini and OaBac7.5mini run described in Figure 7.5:

$MIC_A = MIC_{OaBac5mini} = 1\mu g/mL$ (MIC of OaBac5mini in the right-hand column where the concentration of OaBac7.5 is zero)

$MIC_B = MIC_{OaBac7.5mini} = 2\mu g/mL$ (MIC of OaBac7.5mini in the bottom row where the concentration of OaBac5 is zero)

The FIC is calculated at the point where the two concentrations required are at their lowest as indicated in Figure 7.5.

[A] = [OaBac5mini] = 0.0625μg/mL

[B] = [OaBac7.5mini] = 0.5μg/mL

$$FIC = \frac{[A]}{MIC_A} + \frac{[B]}{MIC_B} = \frac{0.0625}{1} + \frac{0.5}{2} = 0.3125$$

The FIC values indicate whether peptide activities are additive, synergistic or antagonistic. An FIC of unity, i.e. each antimicrobial agent is at half its MIC, indicates that the antimicrobial agents have additive activity. An FIC of 0.5 or less, i.e. each antimicrobial agent at a quarter of its MIC or less, indicates that the antimicrobial agents have synergistic activity. An FIC of four or higher, i.e. each antimicrobial agent at twice its MIC or higher, indicates that the antimicrobial agents are antagonistic.

Three runs were carried out and the results were the same. Each pair of test peptides (SMAP29/OaBac5mini, OaBac7.5mini/SMAP29, and OaBac5mini/OaBac7.5mini) had an FIC of 0.3125. This means that antimicrobial activity was observed when one of the peptides was diluted to one quarter of its MIC and the other peptide was diluted to one sixteenth of its MIC; therefore, they have strong synergistic behaviour.

Synergistic interactions of the peptides *in vivo* would be advantageous and enable the animal's immunity system to deal with most microbial challenges. Firstly, it would mean that the animal would need to produce less of the peptides to fight microbial invasions than would be needed if a single peptide was used. Secondly, it would be more difficult for microorganisms to build up resistance to the peptides because they would be attacked by more than one peptide with different structures and mechanisms of action at the one time.

7.7 SYNERGY BETWEEN PEPTIDES AND KNOWN ANTIBIOTICS

Since the test peptides worked synergistically with each other, it was decided to test if they were also synergistic in conjunction with common antibiotics. The method used is described in Section 3.4.6. For these tests four antibiotics, each with a different mechanism of action, were used. The antibiotics and their mechanisms of action are listed in Table 7.2. The tests were carried out three times and the results were the same. The FICs for each antibiotic in combination with each test peptide are summarized in Table 7.3

Table 7.2 - Antibiotics used in the synergy tests and their mechanisms of actions.

Antibiotic	Mechanism of action
polymyxin B	disrupts the structure/function of the cell membrane
ampicillin	inhibits bacterial cell wall synthesis
kanamycin A	inhibits protein synthesis
rifampicin	inhibits transcription/replication

Table 7.3 - Fractional inhibitory concentrations of synthetic ovine peptides in combination with common antibiotics.

Antibiotic	Fractional inhibitory concentration		
	SMAP29	OaBac5mini	OaBac7.5mini
polymyxin B	0.1875	0.15625	0.28125
ampicillin	1	1	1
kanamycin A	0.53125	0.5625	0.625
rifampicin	0.625	0.5	0.53125

Of the antibiotics tested, only polymyxin B showed strong synergistic activity with all three ovine-derived peptides. The FIC values of polymyxin B in combination with the peptides were low, indicating strong synergistic behaviour. The fractions of the MIC required for activity of polymyxin B and peptide were one sixteenth and one eighth for SMAP29, one eighth and one thirty-second for OaBac5mini, and one quarter and one thirty-second for OaBac7.5mini. In contrast, the FIC values for ampicillin in combination with the peptides were all one, meaning that the activity was additive but not synergistic.

The synergy between polymyxin B and the ovine peptides may be because polymyxin B is also a peptide antibiotic that permeabilises bacterial membranes in a similar way to the ovine peptides (Moore *et al*, 1986). Kanamycin A and rifampicin both interact with the inner cellular macromolecules of the bacteria, so the increased permeability of the cell membranes induced by the ovine peptides may have assisted the uptake of these antibiotics, which is a possible explanation for the increase in activity observed with these two antibiotics. The mechanism used by ampicillin, inhibiting the synthesis of the bacterial cell wall, is unrelated to those used by the peptides, which probably accounts for the lack of synergy shown.

7.8 CONCLUSIONS

The objective of the work presented in this chapter was to determine the effect of different environmental factors on the activity of ovine antimicrobial peptides. Due to their broad-spectrum antimicrobial activity, ovine antimicrobial peptides have the potential to have many useful applications. Before developing applications it was necessary to determine what factors may interfere with, or enhance, the activity of the peptides.

The activities of the test peptides were evaluated in conditions that mimic the physiological properties of raw lamb meat derived from well rested animals, i.e. acidic pH and presence of cations. In these conditions the antimicrobial activity of the peptides were reduced compared

to their activities in low-salt antibiotic testing media (Mueller-Hinton broth). However, the peptides still displayed reasonable antimicrobial activity in the relevant conditions, which indicates that they have the potential to be used as biopreservatives for chilled lamb products.

Further testing is required to determine if the peptides are capable of protecting the meat surface from bacterial contamination. While not guaranteeing the success of these tests, the information gathered in this research suggests that the peptides should be suitable for this application. However, these tests were carried out in liquid media and the peptides may behave differently on a meat surface that will vary considerably in moisture content depending on the chilling regime and the time after slaughter that the peptides are applied. In some processing plants, carcasses are chilled by subjecting them to high velocity air during the initial chilling cycle; whereas, in other plants the carcasses are intermittently sprayed with chilled, chlorinated water and cooled by evaporative cooling of the added water. The peptides could be applied to carcasses that are to be cooled by blast air as they are leaving the cooling floor; whereas, in the case of the spray-chilled carcasses, the peptides may best be applied to the primals just prior to packaging. More work is needed to evaluate the success of these peptides in a variety of chilling and packaging regimes, to assess their viability as potential shelf life extenders for the country's chilled meat exports.

These experiments also showed that the peptides were able to retain their activity when heated to temperatures up to 80°C, but their activity was reduced if they were heated higher than this. This property would not affect the ability of the peptides to be effective chilled meat preservatives, but it may limit their usefulness in applications such as canned products.

The results in this chapter also showed that ovine peptides displayed synergistic activity when used in combination. Due to this, it would be best to use a mixture of peptides in a product as opposed to a single purified peptide. This would also have the advantage of reducing the number of downstream purification steps required to create the product, which would make the process more cost effective. It would also mean that lower concentrations of each of the peptides would be necessary compared to the situations where only single peptides are used.

This study has demonstrated that synthetic ovine antimicrobial peptides are robust and that they retain their activity over a large variety of conditions. The cathelicidins that can be isolated from ovine neutrophils show great potential for a range of applications. These applications should be evaluated, so that it can be decided whether to commercialise the

production of the peptides and hence their use in a range of food products and possibly even pharmaceutical products.

CHAPTER 8
PILOT-SCALE EXTRACTION OF ANTIMICROBIAL PEPTIDES FROM OVINE BLOOD

8.1 INTRODUCTION

The work presented in this chapter was concerned with the fifth objective of this research project, which was to determine whether it is possible to produce an active antimicrobial extract on a scale larger than that used in the laboratory, using industrial-style equipment. The results in the previous chapter showed that the synthetic ovine antimicrobial peptides are robust and can exert antimicrobial activity in a variety of conditions. This indicates that the naturally occurring peptides may be able to be used in products such as biopreservatives and antiseptic topical creams.

The first objective of the research presented in this chapter was to carry out pilot-scale extractions of antimicrobial peptides from ovine blood to determine whether it is possible to produce an active crude neutrophil extract from ovine blood on a pilot-scale. As some of the laboratory scale operations, such as sonication, were not easily scaled up, it was necessary to investigate alternative processes to derive the active peptides.

The second objective was to investigate the activity of the crude neutrophil extract to make sure that the extract produced on the pilot-plant had similar activities to that of the laboratory-derived samples. The MIC of the crude extract was determined against a wide spectrum of organisms. A number of food pathogens and food-spoilage organisms were used to determine if the extract had the potential to be used as a biopreservative for chilled lamb products. The results in Chapter 4, i.e. the production of the antimicrobial peptides using the laboratory procedure, showed that the antimicrobial activity present in the crude solution extracted from ovine neutrophils was predominately caused by the proline/arginine-rich cathelicidin peptides. These peptides were more active against Gram-negative than Gram-positive bacteria and had poor activity against yeast. It was expected that the crude extract produced by the pilot plant process would have similar activity.

To further investigate the activity of the crude extract, TEM was used to see what physical effects the peptides had on microbial cells. The experiments described in Chapter 5 and Chapter 6 showed that the proline/arginine-rich peptides, OaBac5mini and OaBac7.5mini, are

bacteriostatic, not bactericidal, and that they inhibit bacterial cell division by interacting with their inner cellular contents. Therefore, it was expected that the crude extract would not cause notable morphological changes to the cells.

The third objective was to more accurately determine the yield of antimicrobial peptides from ovine blood. Yield is a vital determinant of commercial process viability.

8.2 CRUDE EXTRACTION

A number of extraction runs were carried out using pilot-scale equipment to see if it was possible to produce an active crude extract on a larger scale than was used in the laboratory. Instead of using four litres of blood solution (blood plus sodium citrate solution), which was usual for the laboratory extractions, either 10, 20 or 50 litres of blood solution were processed to more accurately determine the yields. The pilot scale extraction process used for this latter set of experiments is described in Section 3.5.1 and is summarised in Figure 8.1.

There were three main changes in the pilot-scale process from the laboratory process. Firstly an initial centrifugation step was used to remove the blood plasma and the majority of the red blood cells from the pelleted white blood cells in two clean streams. This separation was done using a disc-stack centrifuge (Alfa-Laval Cream Separator) that is usually used for separating milk. A photograph of this centrifuge is given in Figure 8.2.

This centrifugation step had two main advantages. The first advantage was that the blood plasma and the red blood cells were separated into two clean streams. Although this research has focussed on utilising the white blood cells, the economic viability of a venture designed to extract the antimicrobial peptides from neutrophils would be greatly enhanced if the plasma and red blood cell streams were also further processed. The plasma in particular has potential to be converted into high-value products, such as blood serum and blood proteins including serum albumins, fibronectin, transferrin, antibodies and trypsin. In the modified laboratory process the plasma stream was mixed with the lysed blood cells, which would make further processing of the plasma difficult.

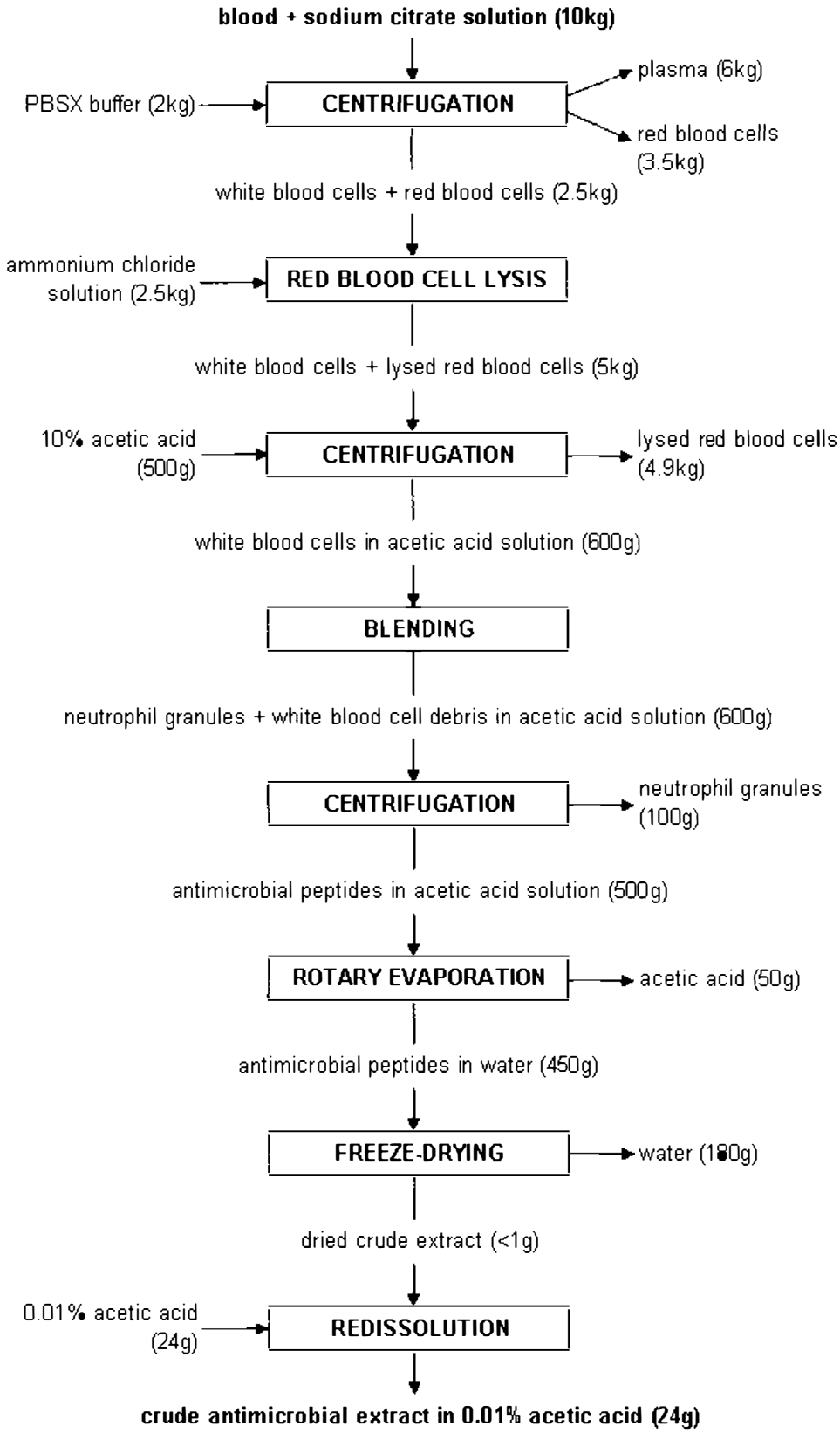

Figure 8.1 - Flow diagram showing the pilot-scale process used to extract antimicrobial peptides from ovine blood. Sodium citrate solution contains 10% sodium citrate. PBSX buffer contains 137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 (pH 7.4). Ammonium chloride solution contains 0.83% ammonium chloride. Acetic acid solution contains 10% acetic acid.

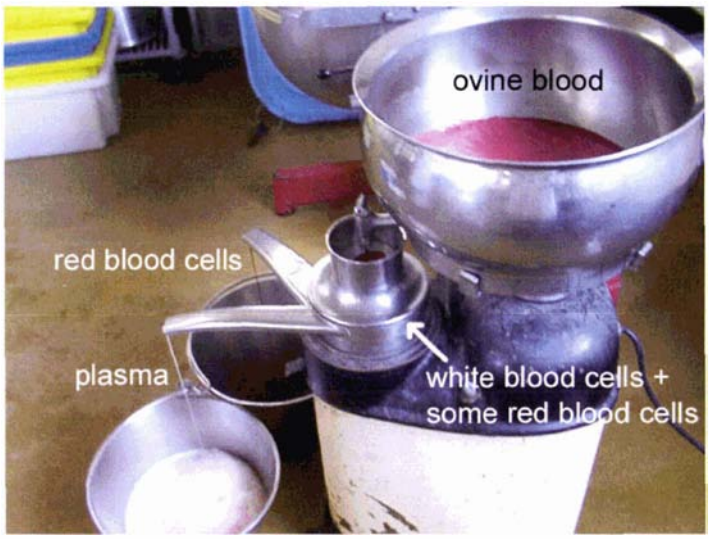

Figure 8.2 - Photograph of the pilot-scale disk-stack centrifuge used to separate white blood cells from plasma and red blood cells. The white blood cells and some of the red blood cells were retained inside the centrifuge on the discs.

The second advantage of the pilot-scale centrifugation process over the laboratory process was that a lot fewer red blood cells needed to be lysed because the majority were removed from the white blood cell fraction during the centrifugation. This reduced the amount of ammonium chloride solution required. In the laboratory process an equal amount of ammonium chloride solution to blood was used, whereas in the pilot-scale process the amount of ammonium chloride solution needed was only a quarter of the volume of the blood processed. This reduction was important because it would reduce the raw material costs of the process and it reduces the amount of ammonium chloride solution that would need to be either recovered or sent to waste. The last course of action would be unfavourable as it would impose undesirable nitrogen loads on the waste treatment system.

The second change in the pilot-scale process compared to the laboratory process was the use of a bench-top blender instead of a sonicator to disrupt the white blood cells. This method was used because it disrupted the cells mechanically instead of using ultrasonic waves. Mechanical breaking of the white blood cells would be more practical in a commercial process because it would not generate as much heat as sonication would on an industrial scale.

The third change in the pilot-scale process compared to the laboratory process was that the white blood cells were resuspended directly in acetic acid solution instead of buffer. This had the benefit of eliminating the need to centrifuge the disrupted white blood cells to collect the neutrophil granules prior to suspension in acid extraction solution.

8.3 MINIMUM INHIBITORY CONCENTRATIONS

The MIC of the crude extract from a typical pilot-scale extraction run was determined using the method described in Section 3.5.2. The crude extract was tested against a variety of microorganisms, including Gram-negative and Gram-positive bacteria, and yeast.

Three runs were carried out, each containing duplicates of each sample. In all cases, at least four of the six recorded values for the MIC of each peptide against each organism were the same. The MIC values varied from the mode values by only a single two-fold dilution. The variations were seen both between duplicates in the same run and between runs. The MICs are reported as the geometric means and the limits of the 95% confidence intervals for the means in Table 8.1. The raw data and example calculations are given in Appendix A4.1.

The MIC values were not expressed as a mass per volume (μg/mL) because the majority of the components in the crude extract did not contribute to the antimicrobial activity. There were variations in the composition of the crude extract between runs so the mass of solids did not necessarily correlate with the antimicrobial activity. To compare activity between extracts, the MIC values were expressed as units/mL. One unit was defined as the amount required to inhibit *E. coli* O111 at a concentration of 10^5 CFU/mL.

The crude extract had very broad-spectrum activity. It was equally active against both the Gram-positive and Gram-negative bacteria. This was unexpected because the purification work in Chapter 4 showed that the majority of the crude extract antimicrobial activity was due to the proline/arginine-rich peptides. In Chapter 5 the synthetic proline/arginine-rich peptides tested were more active against Gram-negative than Gram-positive bacteria. The crude extract was also more active against the yeast than expected. The results presented in Chapter 7 showed that the ovine-derived peptides were strongly synergistic. Therefore, the strong antimicrobial activity of the crude extract may be due to the synergistic interactions between the different peptides present. Alternatively, the strong antimicrobial activity may be due to other peptides or proteins present in the crude extract that were not characterised in Chapter 4

Table 8.1 - Minimum inhibitory concentrations of ovine neutrophil crude extract from the pilot-scale extraction.

Organism	MIC (μL crude/mL total)	MIC (units/mL*)
Gram-negative bacteria		
Escherichia coli 0111	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Escherichia coli 0157:H7	4.5 (3.6, 5.6)	1.1 (0.9, 1.4)
Salmonella enteritidis	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Salmonella typhimurium	7.1 (5.7, 8.9)	1.8 (1.4, 2.2)
Klebsiella pneumoniae	4.5 (3.6, 5.6)	1.1 (0.9, 1.4)
Pseudomonas aeruginosa	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Pseudomonas fluorescens	3.2 (2.0, 5.0)	0.8 (0.5, 1.2)
Yersinia enterocolitica	4.5 (3.6, 5.6)	1.1 (0.9, 1.4)
Gram-positive bacteria		
Staphylococcus aureus NCTC 4163	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Staphylococcus aureus 1056 MRSA	9.0 (7.2, 11.3)	2.2 (1.8, 2.8)
Streptococcus faecalis	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Bacillus cereus	4.5 (3.6, 5.6)	1.1 (0.9, 1.4)
Bacillus nato	5.0 (3.8, 6.7)	1.3 (0.9, 1.7)
Listeria monocytogenes 108 A	2.0 (2.0, 2.0)	0.5 (0.5, 0.5)
Listeria monocytogenes NCTC 10884	2.2 (1.8, 2.8)	0.6 (0.4, 0.7)
Listeria monocytogenes NCTC 7973	4.0 (4.0, 4.0)	1.0 (1.0, 1.0)
Yeast		
C. albicans 3153A	32.0 (32.0, 32.0)	8.0 (8.0, 8.0)

*One unit/mL is defined as the concentration required to inhibit 10^5 CFU/mL *E. coli* 0111.

The MICs are expressed as the geometric means of five runs, each with duplicate samples. The values in the brackets are the lower and upper limits of the 95% confidence intervals of the means. The raw data are given in Appendix A4.1 and calculations are given in Appendix A4.2

Many of the test organisms were food pathogens or food-spoilage organisms. *E. coli* O157:H7, *Listeria monocytogenes, Salmonella enteritidis, Salmonella typhimurium, Yersinia enterocolitica, Streptococcus faecalis, Bacillus cereus* and *Staphylococcus aureus* are all pathogens that can infect food products. Others of the test organisms, such as *Pseudomonas fluorescens* can be involved in food spoilage. The unexpected activity against Gram-negative bacteria as well as its activity against Gram-positive bacteria reinforced the idea that the crude extract had the potential to be an effective preservative for chilled meat products.

Some of the test organisms are clinically significant. *Klebsiella pneumoniae* is common in hospitals where it causes pneumonia and urinary tract infections. *Pseudomonas aeruginosa* is an opportunistic pathogen that causes numerous conditions such as infections of the urinary tract, respiratory system, dermatitis, soft tissue, bone and joints, and gastrointestinal tract. *Candida albicans* is also an opportunistic pathogen that most commonly infects the oral cavity and intestinal tract.

8.4 TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) was used to further investigate the activity of the crude extract. Cultures of *E. coli* O111, *S. aureus* NCTC 4163 and *C. albicans* 3153A were treated with the crude extract and then prepared for TEM according to the method described in Section 3.5.3. For each sample numerous sections were cut and images were taken of random areas within each section. The images presented in Figure 8.3 are typical for each culture.

The majority of the *E. coli* and a large number of the *S. aureus* cells that were treated with crude extract either leaked their inner cellular contents or had completely burst open. Although some of the *E. coli* cells in the control appeared to be damaged, the majority of the cells in both bacteria controls were intact. Therefore, the extensive cell damage seen in the samples treated with the crude extract must be due to the action of the crude extract. Whether the crude extract directly caused this damage, or it weakened the cells so that they lysed during the TEM preparation process, is unknown. The morphological changes observed were more dramatic than those seen with the cultures treated with the individual synthetic ovine peptides in Sections 6.2 and 6.3.

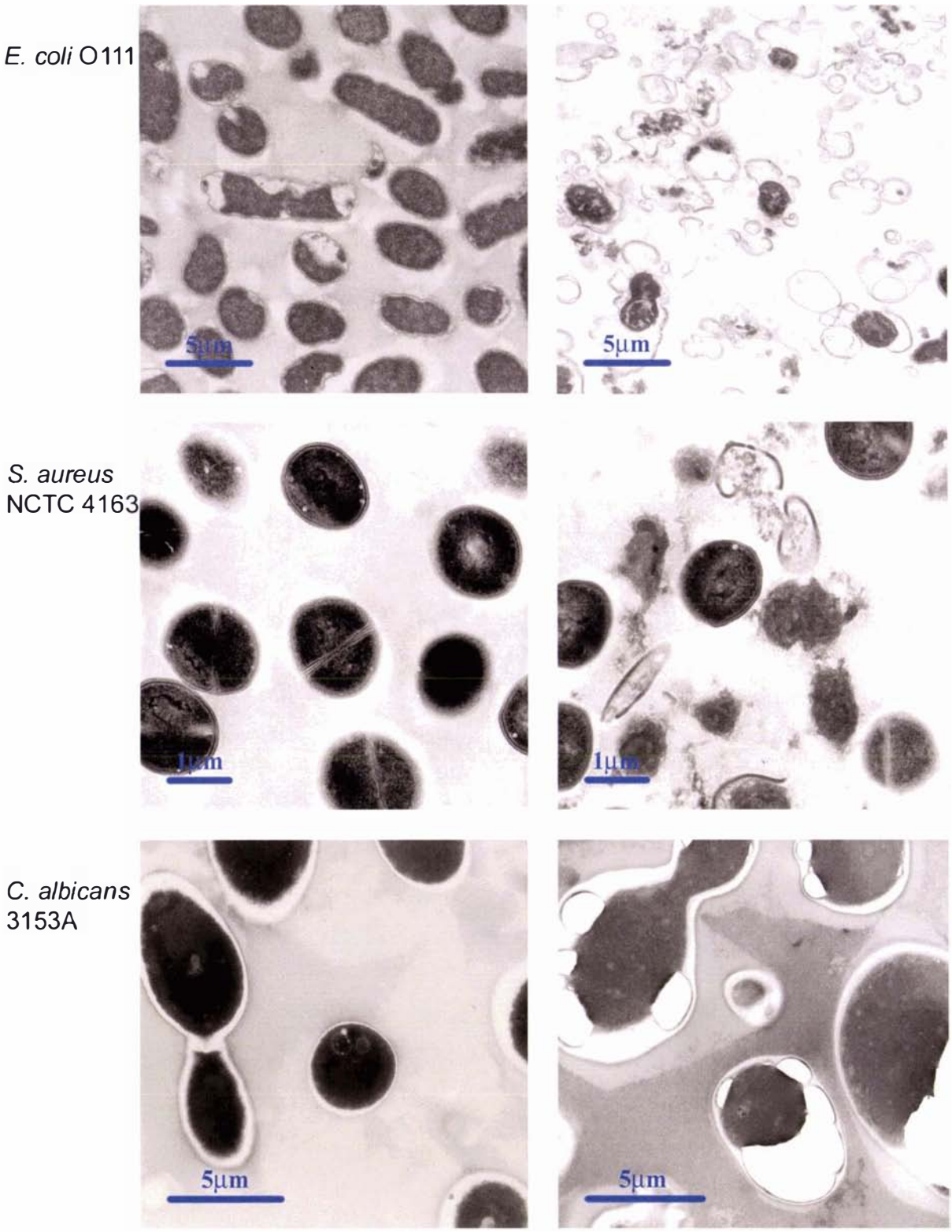

Figure 8.3 - Transmission electron microscopy images of control cells treated with 0.01% acetic acid (left) and cells treated with ovine neutrophil crude extract (right).

The majority of the yeast cells that were treated with the crude extract appeared to be damaged. They were not leaking their inner cellular contents or burst open as was seen with the bacterial cells. Instead they had large areas where the outer membrane appeared to be peeled off the cell wall and air sacs or vacuoles were present. Due to the thick cell wall of the yeast, the cells held together; however, perturbation of the cell wall and cell membrane allowed fluid or air to accumulate inside the cells. The control cells did not have these ultrastructures so they must have been induced by the crude extract.

Like the MIC results, these TEM results were unexpected. The purification work in Chapter 4 showed that the crude extract activity was predominately due to the proline/arginine-rich peptides, but the mechanisms and microscopy work in Chapter 5 and Chapter 6, using synthetic proline/arginine-rich peptides, showed that these peptides did not induce cell death and had little effect on cell morphology. Again, this may be due to the peptides acting synergistically, or to the presence of other peptides or proteins that were not characterised.

8.5 YIELD CALCULATION

For each of the pilot-scale extraction runs the yield of units of activity per litre of blood was calculated using the method described in Section 3.5.4.

The calculation of the yield for a typical pilot-scale extraction was done as follows:

Starting volume	= 10L of blood solution
	(9L of whole blood + 1L 10% sodium citrate)
Final volume	= 24mL of crude extract
MIC against *E. coli* O111	= 1μL crude extract/100μL solution
	= 10μL crude extract/mL solution

If 1 unit of activity is described as the amount required to inhibit 1mL of 1×10^5 *E. coli* O111 then for this extract:

1 unit	= 10μL extract

Therefore:

Total units extracted	= 24mL/10μL
	= 2400units

Therefore:

Yield = 2400units/9L whole blood

= 267units/L whole blood

The calculated yields of the pilot-scale extractions are summarised in Table 8.2. For the pilot-scale extractions the yield varied between 260 and 300units/L of whole blood. In comparison, the laboratory process yield was usually between 300-360units/L of blood. Therefore, the current pilot-scale extraction process is not as effective as the laboratory process, so process optimisation is required to reduce the losses and increase the yield.

Table 8.2 - Yields for pilot-scale extractions of antimicrobial peptides from ovine blood.

Run	Blood volume (L)	Crude extract volume (mL)	MIC (μL crude extract/mL)	Yield (units/L blood)
1	9	24	10	267
2	18	23.5	5	261
3	18	27	5	300
4	45	65	5	289

From the MIC results it is apparent that a concentration of 3units/mL would be sufficient to inhibit most bacteria *in vitro*. With a yield of 260-300units/L of blood it would be possible to produce 87-100mL of crude extract at a concentration of 3unit/mL for each litre of blood processed. However, further experiments are required to determine what concentration of extract is required in practical applications.

8.6 INDUSTRIAL-SCALE PROCESS

The pilot-scale extraction trials showed that it was possible to effectively extract a crude antimicrobial extract from ovine blood on a scale larger than that done in the laboratory. If the product were to be made for commercial sale, the process would need to be scaled-up and modified further. The appropriate industrial equipment for each step was investigated. From the original laboratory extraction process, which contained eleven steps, the process was reduced to six necessary steps for an industrial-scale extraction. These steps are outlined in Figure 8.4.

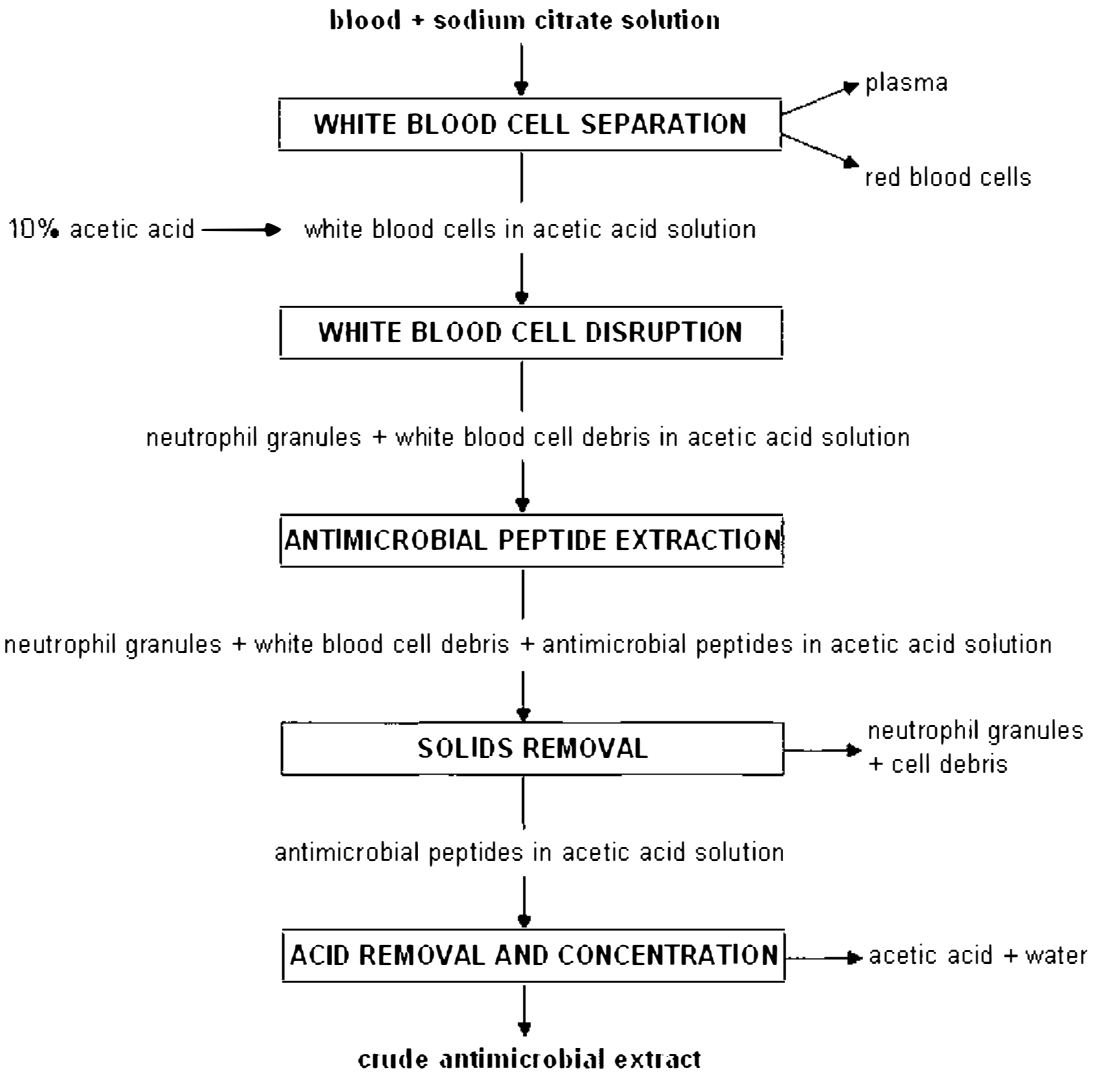

Figure 8.4 - Steps in an industrial process to produce a crude antimicrobial extract from ovine blood.

The first step in the process is to remove any solids from the incoming blood stream. Commonly pieces of wool and clotted blood are present in the collected blood. As in the laboratory, in an industrial process these solids could be removed using a filter screen.

The next step is to separate the white blood cells from the blood plasma and the red blood cells. In the laboratory process the red blood cells were lysed using ammonium chloride solution, then the white blood cells were collected using centrifugation. In the pilot-scale process the white blood cells were separated from the plasma and the majority of the red blood cells using centrifugation, then the remaining red blood cells were lysed with ammonium chloride solution, to remove them from the white blood cell fraction.

Ideally it would be best to collect the white blood cells in single step. The clean separation of the neutrophils from the red blood cells was not possible with the pilot-scale centrifuge used for this research because it did not have adjustable rotational speed. However, the calculations in Appendix A4.3 show that it would be possible to design a centrifuge to do this separation. This would be advantageous because it would eliminate the need for a lysis step to remove contaminating red blood cells from the white blood cell fraction.

The third step in the process is the disruption of the white blood cells to release the neutrophil granules. In the laboratory this was achieved using sonication or a bench-top blender. In an industrial-scale process numerous other mechanical processes could replace this operation (Meat New Zealand, 2003), such as an homogeniser that uses high pressure pumping through a tiny orifice or a mechanical grinding technique (Perry and Green, 1997).

The next step in the process is to extract the antimicrobial peptides from the neutrophil granules. In the laboratory this was carried out using 10% acetic acid with continuous mixing. Instead of using a mixing vessel that would result in a batch process, there are a number of extraction vessel designs that can be used in a continuous process (Braga and Ricci, 1998). These are usually run counter-currently. This means that the fresh solution comes in contact with the most spent solids, which increases the total amount extracted. Further experiments are required to determine the type of extraction vessel and most suitable arrangement.

After the extraction has been carried out the next step is to remove the granules and cell debris from the solution. In the laboratory this was achieved using centrifugation, but in an industrial process a filter press could be used instead. The insoluble solids would be retained and the clarified solution containing the antimicrobial peptides would pass through.

The final step in the process is to remove the acid and concentrate the solution ready for use. In the laboratory this was carried out using rotary evaporation to remove the acid and freeze-drying to remove the water. The dried solids were then resuspended in 0.01% acetic acid for use. As an alternative, in an industrial process diafiltration, which concentrates and washes the suspended proteins/peptides, could be used. This involves passing the solution through an ultrafiltration membrane with a low molecule weight cut-off. The peptides of interest are approximately 3kDa so a cut-off of 0.5kDa would be appropriate in this case. The retained proteins and peptides would then be washed with water, i.e. diafiltration, to remove any salts.

As well as the number of steps required, another factor that was reduced from the laboratory process to the industrial-scale process was the number and amounts of chemicals required. The industrial process does not require ammonium chloride solution or PBSX buffer, both of which were used for the lab extractions. This eliminates the need to purchase and dispose of these chemicals.

8.7 CONCLUSIONS

The first objective of the research presented in this chapter was to carry out pilot-scale extractions of antimicrobial peptides from ovine blood to determine whether it is possible to produce an active crude neutrophil extract from ovine blood on a pilot-scale. The results showed that an active crude extract could be produced from ovine blood on a pilot-scale. It also showed that the process worked effectively using industrial-style equipment instead of laboratory equipment.

The preliminary industrial process design showed that it should be possible to produce an active crude extract on an industrial-scale using six simple unit operations and minimal chemicals. Further pilot-scale trials are required to determine what equipment and conditions are optimal.

The most important simplification to the laboratory-scale process was the white blood cell separation step. In the laboratory process the red blood cells were lysed using ammonium chloride solution, then the white blood cells were isolated using centrifugation. In the pilot-scale process the whole blood was centrifuged first and the plasma and the majority of the red blood cells were separated from the white blood cells in two clean streams. In an industrial process it will be possible to specify a centrifuge that was able to cleanly separate all the white blood cells from the red blood cell and plasma streams. This would have the advantages of allowing the red blood cells and plasma streams to be further processed into other products and it would eliminate the use of ammonium chloride solution.

The second objective the work presented in this chapter was to investigate the activity of the crude neutrophil extract to make sure that the extract produced on the pilot plant had similar activities to that of the laboratory derived samples. The MIC results showed the crude extract had potent activity against Gram-negative and Gram-positive bacteria, including numerous food pathogens and clinically significant organisms, and was relatively active against the yeast *C. albicans*. This was unexpected because the major active molecules in the crude

extract were the proline/arginine-rich peptides that were shown to be more active against Gram-negative than Gram-positive bacteria, and poorly active against yeast.

The TEM results showed that the crude extract induced lysis of Gram-positive and Gram-negative bacteria and caused the separation of the membrane from the yeast cells. Again these results were unexpected because they did not correspond to the properties of the synthetic proline/arginine-rich peptides that were evaluated. The proline/arginine-rich peptides were not bactericidal individually and did not induce morphological changes on bacterial cells.

The enhanced activity of the crude extract compared to the individual peptides may have been due to synergistic interactions between the peptides, or due to a component in the crude extract that was not characterised. This shows that it would be better to use the crude extract in a product as opposed to purified peptides. Further purification would be costly on an industrial-scale and would mostly likely reduce the effectiveness of the product.

The third objective of the work presented in this chapter was to more accurately determine the yield of antimicrobial peptides from ovine blood. The results of this research also showed that the yield of antimicrobial activity was 260-300 units/L of blood. This means that from each litre of blood that was processed it was possible to make 87-90mL of crude extract at a concentration three times that required to inhibit 10^5 CFU/mL *E. coli* O111. This is a sufficient concentration to inhibit all the bacteria tested *in vitro*.

The next step should be to develop applications for the crude extract, such as coating for chilled-lamb products or as a component in topical creams. Once it has been determined what concentration of the crude extract would be required for a given application or product, then the cost for producing the product should be estimated. From this, it will be possible to determine whether the process is economically viable.

CHAPTER 9
CONCLUSIONS AND RECOMMENDATIONS

9.1 SUMMARY OF RESEARCH CONCLUSIONS

The first objective of the research presented in this thesis was to purify and identify antimicrobial peptides from ovine blood. Prior work had shown a crude extract from ovine neutrophils had antimicrobial activity. It was hypothesised that the activity was due to antimicrobial peptides, because the literature review uncovered that seven ovine antimicrobial peptides were predicted from ovine cDNA. However, only one of these had been purified from ovine blood.

In the research presented in this thesis, a number of peptides responsible for the antimicrobial activity of the crude extract were identified. The majority of these were proline/arginine-rich cathelicidin peptides. OaBac5 and its variant OaBac5γ were both characterised. Prior to this work, OaBac5 had been identified only from cDNA, and two variants, OaBac5α and OaBac5β, had been isolated from ovine blood. Other proline/arginine-rich peptides were also characterised, including a truncated form of OaBac7.5 and various truncated forms of OaBac11. Both OaBac7.5 and OaBac11 were previously predicted from ovine cDNA, but neither of them had been isolated from ovine blood.

As well as the proline/arginine-rich cathelicidins, two other novel peptides that displayed antimicrobial activity were isolated from the crude ovine neutrophil extract. One of these peptides was similar to part of the cathelin domain of the cathelicidin peptides. Previously it was thought that this domain was responsible for suppressing the antimicrobial activity of the peptides until they were cleaved for use. Now it appears that this domain may also play a role in inhibiting microorganisms. The other peptide identified was part of the signal peptide of the T-cell glycoprotein CD4 precursor. This peptide was very small but it had relatively high antimicrobial activity.

The second objective of the research presented in this thesis was to determine the mechanisms of action of ovine antimicrobial peptides. The literature review revealed that antimicrobial peptides had different mechanisms of action depending on their structures. Therefore, this research compared the mechanisms used by peptides from different structural classes.

For this research three synthetic ovine peptides were used. One of these peptides, SMAP29 was α-helical and the other two, OaBac5mini and OaBac7.5mini, were proline/arginine-rich peptides. SMAP29 had potent broad-spectrum activity. OaBac5mini and OaBac7.5mini were less active than SMAP29, but were more active than their bovine homologues.

The research presented in this thesis showed that the α-helical peptide had a different mechanism of action to the proline/arginine-rich peptides. The membrane interaction studies showed that all three test-peptides were able to bind to bacterial surface LPS, permeate the outer membrane, and interact with the cytoplasmic membrane of Gram-negative bacteria. However, the way they interacted with the cytoplasmic membrane differed. SMAP29 caused significant depolarisation of the cytoplasmic membrane; whereas, the other two peptides only induced slight depolarisation.

Other studies were also carried out to investigate the mechanism of action of the synthetic ovine peptides. Optical density and viable cell count studies showed that SMAP29 caused a rapid decrease in the number of viable cells and a slight decrease in optical density of the culture. This meant that the depolarisation of the cytoplasmic membrane caused by SMAP29 was the lethal event and probably led to lysis of the cells. In contrast, OaBac5mini and OaBac7.5mini did not decrease the number of viable cells or the optical density of the culture; instead they limited the growth of the cells. This meant that they were bacteriostatic but not bactericidal. It was predicted that the proline/arginine-rich peptides inhibited cells by passing across the cytoplasmic membrane and interacting with the inner cellular contents. Further studies showed that these peptides were able to bind to DNA, so this may be their mechanism of action. However, SMAP29 was also able to bind to DNA, which indicates it may have more than one mechanism of inhibiting cells.

The third objective of the research presented in this thesis was to investigate the morphological changes to microbial cells induced by ovine antimicrobial peptides. The literature review showed that antimicrobial peptides induce different morphological changes to microbial cells depending on their mechanism of action. Therefore, the aim of this research was to compare the morphological changes to bacterial cells induced by the synthetic ovine peptides.

The research presented in this thesis showed that SMAP29 induced substantial morphological changes to bacterial cells; whereas, OaBac5mini did not. Microscopy studies using TEM

confirmed that SMAP29 did act on the cytoplasmic membrane and induced cell lysis; not only to Gram-negative cells, but also to Gram-positive cells. In contrast, OaBac5mini did not induce cell lysis, but it did cause the cell membrane to separate from the cytoplasmic membrane in parts of some cells. AFM was also used to image the surface of cells treated with the peptides; however, this technique was not very successful for this application.

The fourth objective of the research presented in this thesis was to determine the effect of different environmental factors on the activity of ovine antimicrobial peptides. The literature review showed that some antimicrobial peptides were inhibited by high salt concentrations, divalent cations and acidic pH values, and some peptides were not. The aim of this research was to determine the effects of a variety of conditions on the synthetic ovine peptides.

The research presented in this thesis found that the ovine antimicrobial peptides were very robust. The activity of the peptides was reduced in physiological conditions, such as in the presence of cations and at acidic pH; however they were still relatively active. The peptides were also resistant to heat. They showed no decrease in activity when heated to temperatures up to 80°C, and had only a slight decrease in activity when heated to temperatures above this, or when autoclaved.

To further investigate the effect of different conditions on the antimicrobial peptides, synergy studies were carried out. This showed that the ovine antimicrobial peptides worked significantly better in combination with each other than alone. This means that it would be better to use a mixture of peptides in a product, as opposed to a single purified peptide. This would have the advantage of reducing the number of downstream process purification steps required to create the product, and reducing the concentration of the peptides required, both of which would make the process more cost effective.

The fifth objective of the research presented in this thesis was to determine whether it is possible to produce an active antimicrobial extract on a scale larger than that used in the laboratory, using industrial-style equipment. The literature search did not find any previous cases where antimicrobial peptide had been extracted on a large scale.

The research presented in this thesis showed that it was possible to produce the antimicrobial extract on a scale larger than that in the laboratory using equipment similar to that found in industrial processes. A number of successful pilot-scale trials were carried out. The

laboratory process was simplified from eleven unit operations to only six necessary unit operations.

For each pilot-scale extraction the yield was determined. The yields were between 260 and 300 units per litre of blood, which is sufficient to make 87 to 90mL of crude extract at a concentration three times that required to inhibit 10^5 CFU/mL *E. coli* 0111 from each litre of blood. This concentration was chosen because the crude extract was active against all the food pathogens tested at either equal to, or two-fold higher than, the MIC for *E. coli* 0111.

The MIC and TEM tests carried out with the crude extract confirmed that it would be best to use the peptides in a mixture. The crude extract had higher and more broad-spectrum activity than expected and was able to induce cell lysis, even though it consisted of predominately proline/arginine-rich cathelicidins that do not display these properties in their pure forms. It is unclear whether this was due to the peptides acting synergistically or to other components present in the crude extract that were not characterised.

9.2 RECOMMENDATIONS FOR FUTURE RESEARCH

Although the results presented in this thesis yielded a lot of useful information, the results also uncovered more unanswered questions. Areas where further fundamental research on ovine antimicrobial peptides could be carried out include:

1) Investigating the reason for the variations in the OaBac5 sequence. To date, four different sequences have been identified. These variations may be due to each animal having multiple copies of the OaBac5 gene that vary slightly, or they may be due to genetic differences between animals because the blood was collected from more than one animal.

2) Investigating the reason for OaBac7.5 and OaBac11 truncations. Both of these peptides have been isolated only in truncated forms. This is probably due to enzymatic cleavage of the peptides during the extraction process, but the enzymes responsible have not yet been identified.

3) Investigating the reason why the other predicted ovine cathelicidins peptides, especially the potent SMAP29, were not isolated from the neutrophil extract. This may be because these peptides are not expressed constitutively. It is possible that they are produced only when infection occurs. Alternatively, these peptides may be expressed in different parts of the animal instead of the blood.

4) Investigating the reason for the different mechanisms of action used by the different peptides. This may provide insurance against an infection, because if one mechanism is not successful, then the other may still be able to inhibit the invading organism. SMAP29 itself may also be able to use more than one mechanism of action, but further work is required to confirm this.

5) Investigating the use of scanning electron microscopy to image the surface of treated cells. This technique should give information similar to that expected from the atomic force microscope; however, it may be less technically difficult.

6) Investigating other properties of the peptides. Some other antimicrobial peptides have proven to have antiviral, anti-tumour and anti-inflammatory activity, so these areas should also be studied.

The results presented in this thesis indicated that ovine antimicrobial peptides may be suitable for use in commercial products. Areas where further research into the possible commercialisation of the crude antimicrobial extract could be carried out include:

1) Investigating the effectiveness of the crude antimicrobial extract as a biopreservative for chilled lamb products. This research showed that the antimicrobial peptides retained their activity in a variety of conditions, which indicates that they may be useful as a biopreservative. However, further studies are required to determine its efficacy. The best way to apply the extract to the meat cuts should also be tested.

2) Investigating the use of the crude antimicrobial extract as a topical cream. This could be an antiseptic cream for cuts and grazes, an anti-fungal cream for infections such as athletes' foot, or an anti-inflammatory cream for conditions such as arthritis. The antimicrobial extract could also be added to existing products, such as lanolin cream, to enhance the product and add value.

3) Investigating the various types of industrial-scale equipment available to carry out each step in the extraction process. Further pilot-scale trials are required to determine the most effective process for producing the crude extract. It is important that the process is optimised so that the maximum amount of product can be produced. More accurate cost analysis will be possible once the best industrial process has been defined.

4) Investigating the possible products that can be produced from the blood plasma and red blood cells fractions. Further process development and cost analysis is also required to include these product streams.

9.3 FINAL CONCLUSION

The overall objective of this research project was to gain an understanding of the antimicrobial peptides that occur naturally in ovine blood, so that their potential to be utilised in high-value products could be assessed. This research has shown that the antimicrobial peptides found in the crude neutrophil extract produced from ovine blood have stable, broad-spectrum activity, and that they have the potential to be effective biopreservatives and topical antiseptic agents. This research also showed that the peptides were significantly more active in combination than alone, which indicates that the use of the peptides as a mixture would be best. Finally, this research showed that is was possible to produce the crude antimicrobial peptide on a pilot-scale and that the industrial-scale process required would be relatively simple. Therefore, it is recommended that further work be carried out to develop the extraction process further and to investigate the applications of the peptides with the aim of commercialisation in the future.

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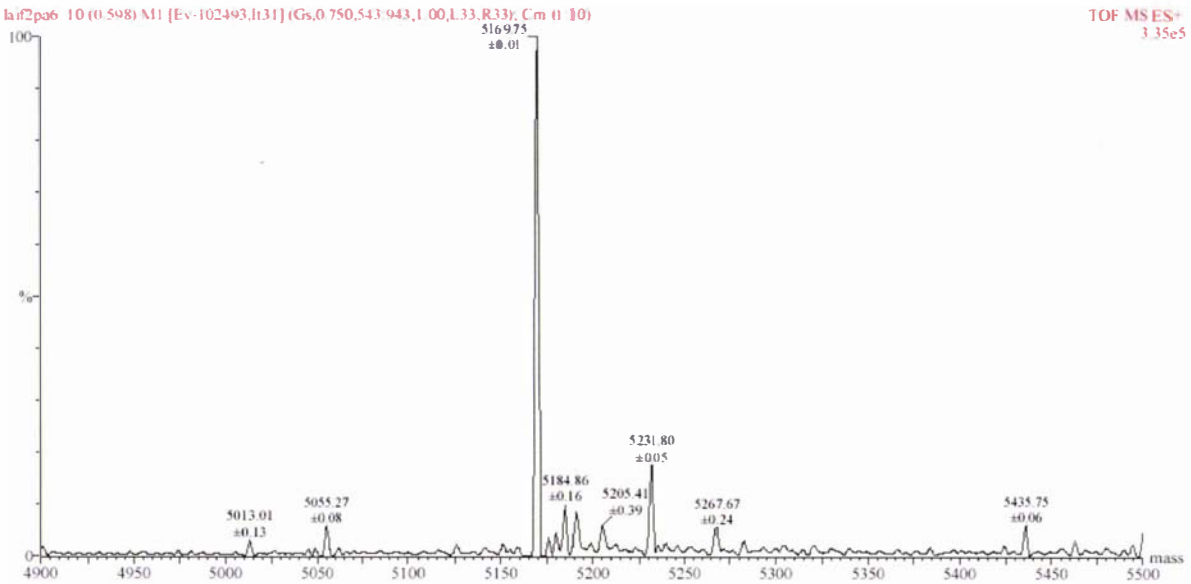
APPENDIX A1

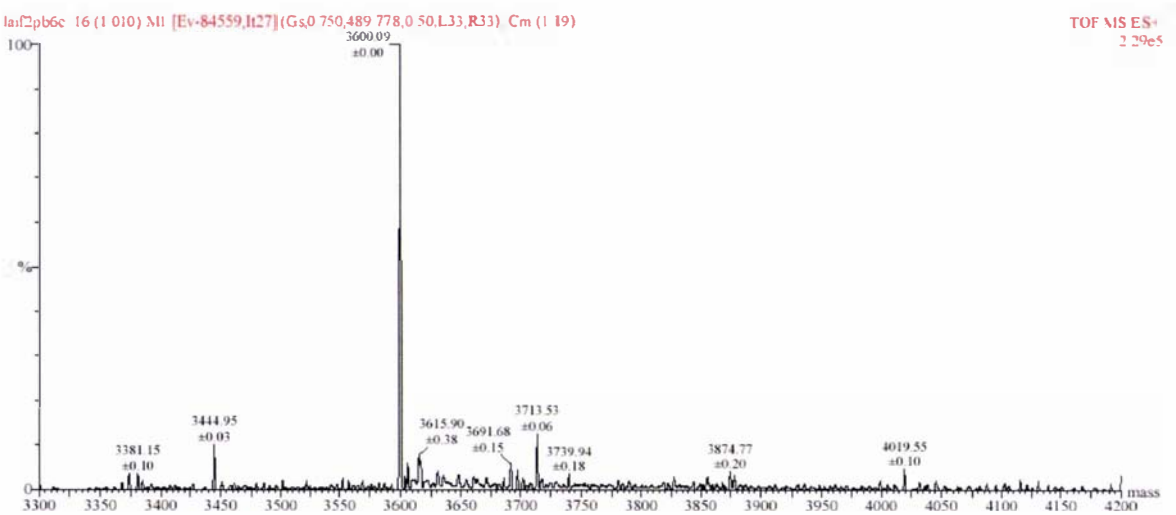
RAW DATA AND CALCULATIONS FROM CHARACTERISATION STUDIES

A1.1 MASS SPECTRA OF THE PURIFIED HPLC PEAKS

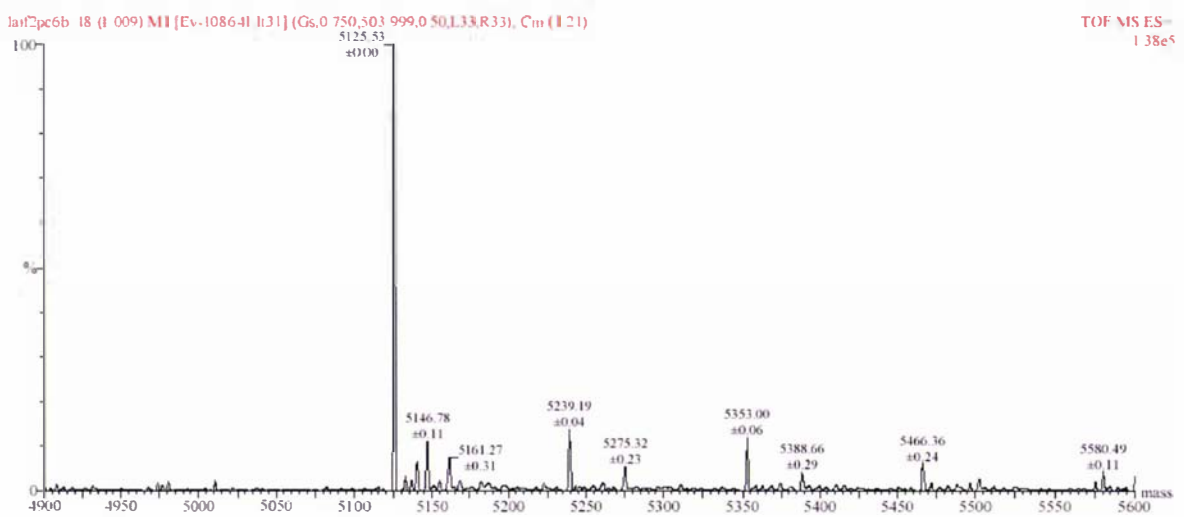
HPLC Pa from gel filtration F2 (OaBac5)



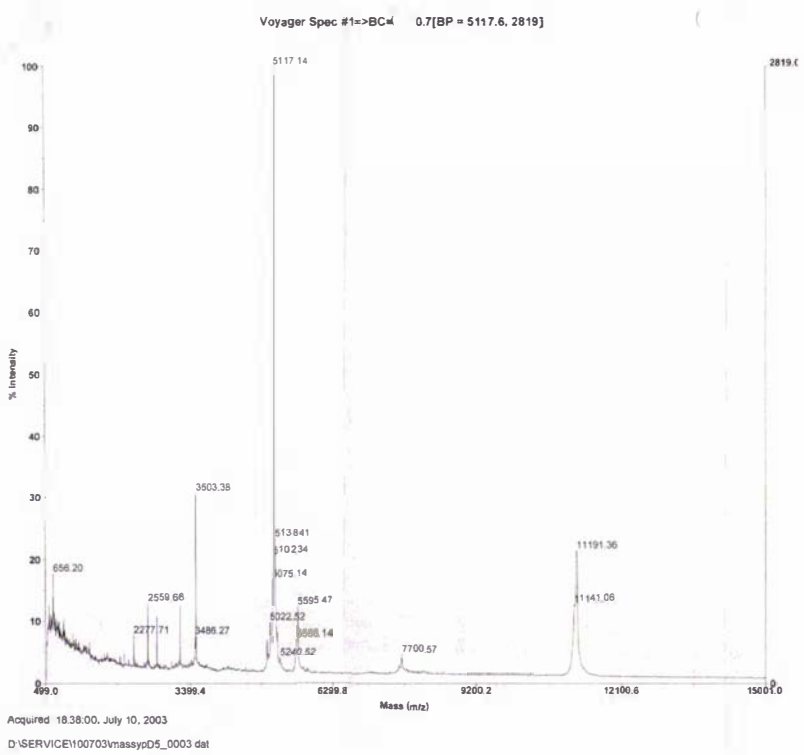
HPLC Pb from gel filtration F2 (OaBac7.5$_{(32\text{-}60)}$)



HPLC Pc from gel filtration F2 (OaBac5γ)



HPLC Pd from gel filtration F2 (OaBac11 truncates)



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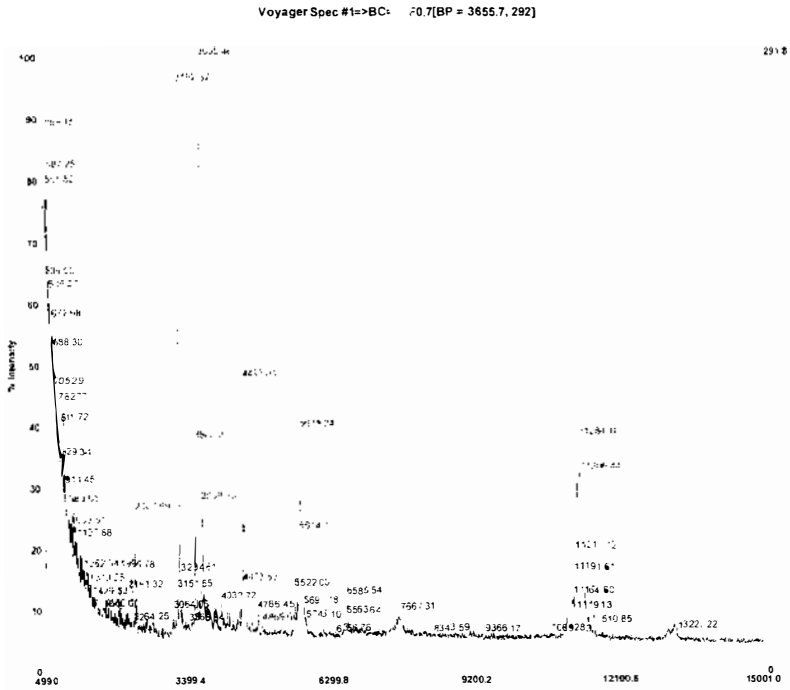
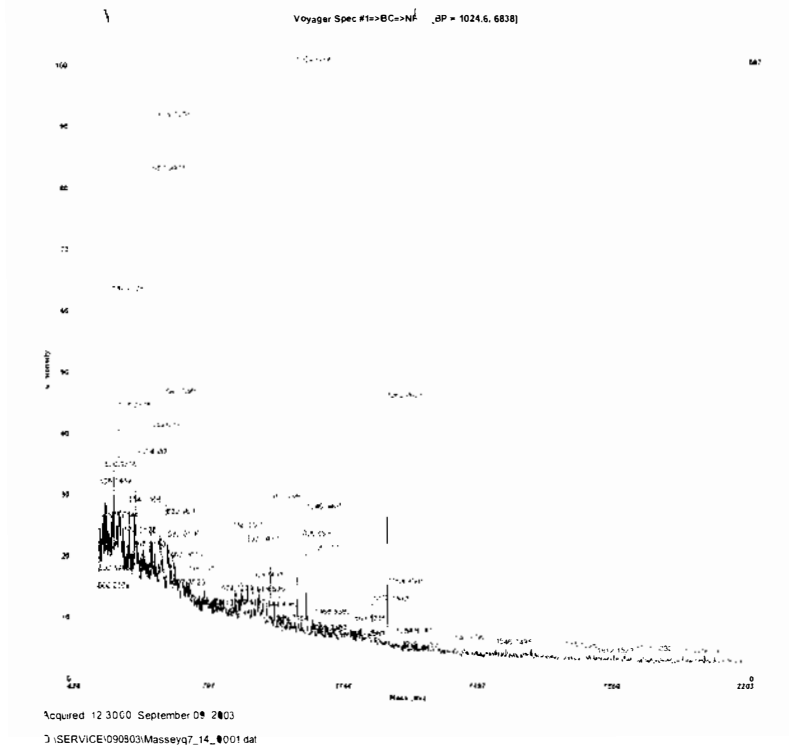


HPLC P14 from cationic fraction (unidentified)



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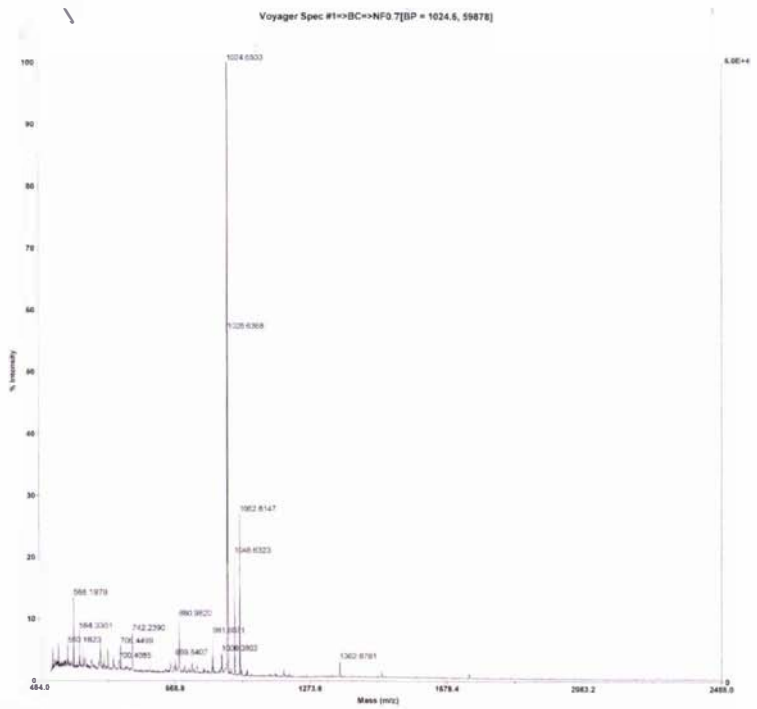

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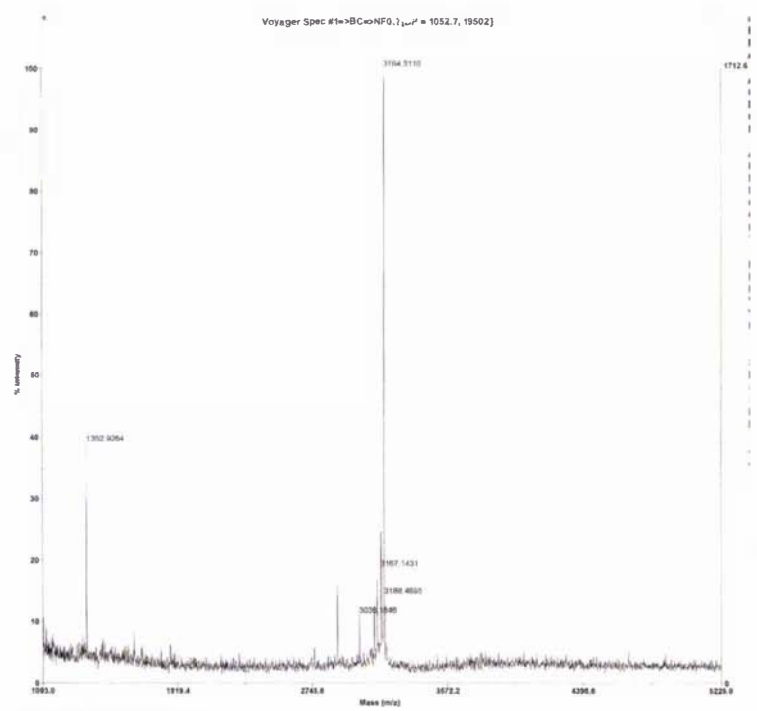
HPLC P18 from cationic fraction (Fragment of Cathelin domain)



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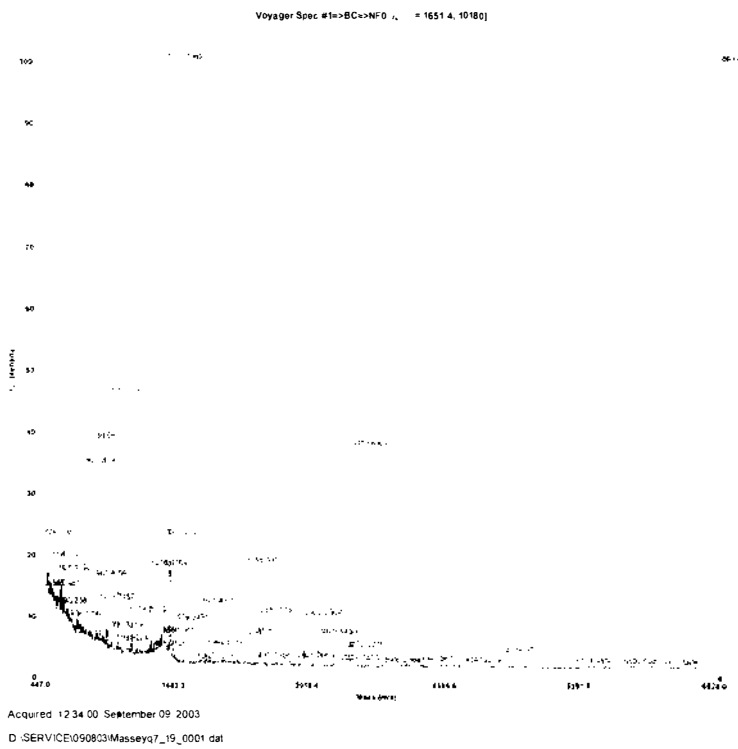
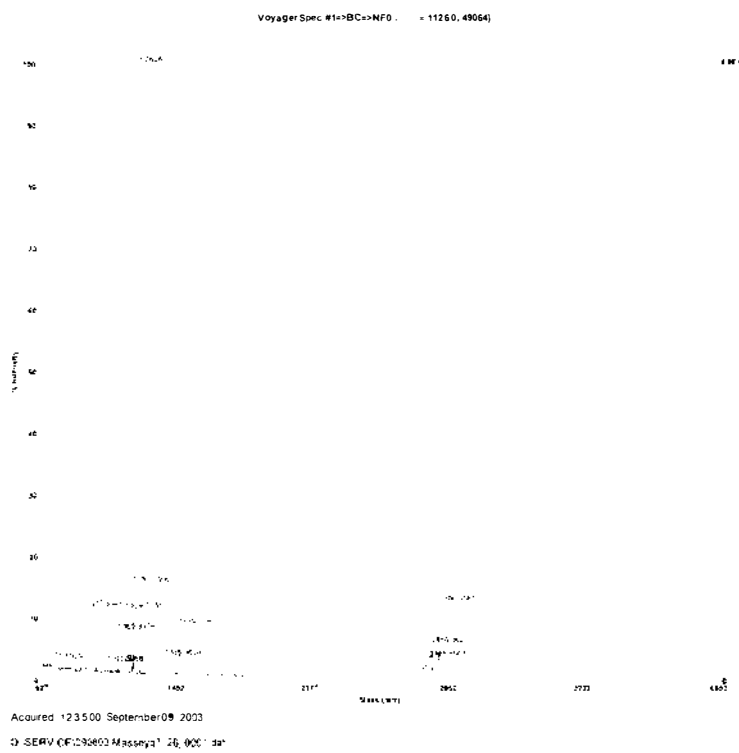
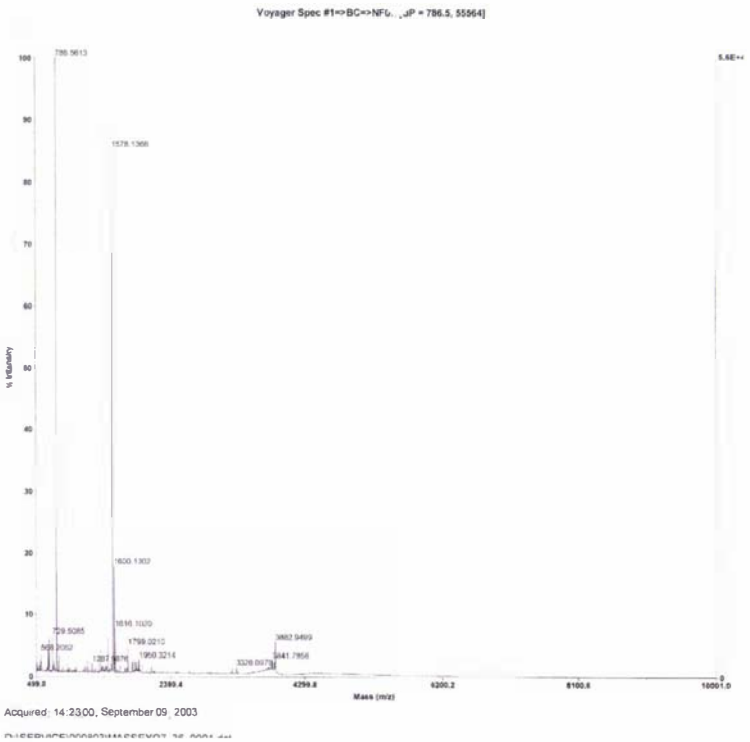
HPLC P19 from cationic fraction (unidentified)



HPLC P24 from cationic fraction (fragment of T-cell surface protein CD4 precursor)



HPLC P32 from cationic fraction (unidentified)



A1.2 EXAMPLE CALCULATION OF CONFIDENCE INTERVALS FROM PLATE ASSAY RAW DATA

The calculation of the MIC of Pa (OaBac5) from the raw plate assay data was carried out as described below. The raw data from the plate assay of Pa against *E.* coli O111 are given below.

Dilution	Conc (μg/mL)	Clearing (mm)	Ln conc
1	25.00	2.00	3.22
1/2	12.50	1.00	2.53
1/4	6.25	0.50	1.83
1/8	3.13	0.00	1.14

The log concentrations and the clearing sizes were entered into the statistics package GenStat. Linear regression analysis was done by entering the clearing size as the response variate and the Ln conc as the constant. The GenStat output is shown below.

```
***** Regression Analysis *****

 Response variate: clearing
     Fitted terms: Constant, ln_conc


*** Summary of analysis ***

              d.f.         s.s.         m.s.      v.r.  F pr.
Regression       1      2.11250      2.11250     56.33  0.017
Residual         2      0.07500      0.03750
Total            3      2.18750      0.72917

Percentage variance accounted for 94.9
Standard error of observations is estimated to be 0.194


*** Estimates of parameters ***

                 estimate         s.e.      t(2)  t pr.
Constant           -1.169        0.289     -4.04  0.056

ln_conc             0.938        0.125      7.51  0.017
```

From the GenStat output the y-intercept (B_0), the gradient (B_1) and the residual ms (S^2) were recorded. In this case B_0=-1.169, B_1=0.938 and S^2=0.03750.

The MIC was the point where the clearing size is zero so it is equal to the x-intercept. The x-intercept was calculated for the equation of the line as follows:

$y = 0.9838x + -1.169$

when y=0

x = 1.169/0.9838 = 1.246 (this is the Ln MIC)

MIC = exp(1.246) = 3.476 μg/mL

The 95% confidence intervals for the x-intercept were calculated using the formula below.

$$\text{Limits of confidence intervals} = g(\hat{X}_0 - \bar{X}) \pm \frac{t}{b_1}\sqrt{s^2}\sqrt{\frac{(\hat{X}_0 - \bar{X})}{Sxx} + \frac{(1-g)}{n}}$$

where:

$$g = \text{constant} = \frac{t^2 s^2}{b_1 Sxx}$$

$\hat{X}_0$ = the estimated x-intercept

$\bar{X}$ = the mean of the x values

t = the t-value

b_1 = gradient of the line-of-best-fit

s^2 = estimate of the pooled variance

Sxx = total corrected sum of the squares for x = $(X_1 - \bar{X})^2 + (X_2 - \bar{X})^2 + (X_3 - \bar{X})^2 + (X_4 - \bar{X})^2$

n = the number of x values

A1.3 RAW DATA, CALCULATED MICS AND 95% CONFIDENCE INTERVALS FOR THE MICS OF THE PURIFIED PEPTIDES

MIC of Pa

***E.coli* 0157:H7**

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc		
1	25.00	2.00	3.22	xmean	2.18
1/2	12.50	1.00	2.53	ymean	0.88
1/4	6.25	0.50	1.83	Sxx	2.40
1/8	3.13	0.00	1.14		
bo	-1.17	Ln mean	1.25	**mean**	3.48
bi	0.94	Ln upper limit	-0.17	**upper limit**	0.84
s2	0.04	Ln lower limit	1.75	**lower limit**	5.77

***S. aureus* 0156 MRSA**

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc		
1	30.00	0.75	3.40	3.05	
1	30.00	0.25	3.40	0.31	
1/2	15.00	0.25	2.71	0.48	
1/2	15.00	0.00	2.71		
bo	-1.35	Ln mean	2.49	**mean**	12.02
bi	0.54	Ln upper limit	n/a	**upper limit**	n/a!
s2	0.08	Ln lower limit	n/a	**lower limit**	n/a

***C. albicans* 3153A**

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc		
1	30.00	0.00	3.40		
1/2	15.00	0.00	2.71		
1/4	7.50	0.00	2.01		
1/8	3.75	0.00	1.32		
bo	n/a	Ln mean	n/a	**mean**	>30
bi	n/a	Ln upper limit	n/a	**upper limit**	n/a
s2	n/a	Ln lower limit	n/a	**lower limit**	n/a

***E.coli* 0157:H7 with 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc		
1	25.00	0.00	3.22		
1/2	12.50	0.00	2.53		
1/4	6.25	0.00	1.83		
1/8	3.13	0.00	1.14		
bo	n/a	Ln mean	n/a	**mean**	>25
bi	n/a	Ln upper limit	n/a	**upper limit**	n/a
s2	n/a	Ln lower limit	n/a	**lower limit**	n/a

***S. aureus* 1056 MRSAwith 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc		
1	30.00	0.00	3.40		
1/2	15.00	0.00	2.71		
1/4	7.50	0.00	2.01		
1/8	3.75	0.00	1.32		
bo	n/a	Ln mean	n/a	**mean**	>30
bi	n/a	Ln upper limit	n/a	**upper limit**	n/a
s2	n/a	Ln lower limit	n/a	**lower limit**	n/a

C. albicans 3153A with 100mM NaCl

Dilution	Conc (ug/mL)	Clearing (mm)	Ln conc
1	30.00	0.00	3.40
1/2	15.00	0.00	2.71
1/4	7.50	0.00	2.01
1/8	3.75	0.00	1.32

bo	n/a	Ln mean	n/a	**mean**	>30
bi	n/a	Ln upper limit	n/a	**upper limit**	n/a
s2	n/a	Ln lower limit	n/a	**lower limit**	n/a

MIC of Pb

E.coli 0157:H7

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	30.00	2.25	3.40	3.05	xmean
1	30.00	1.75	3.40	1.25	ymean
1/2	15.00	0.75	2.71	0.48	Sxx
1/2	15.00	0.25	2.71		

bo	-5.39	Ln mean	2.48	**mean**	11.93
bi	2.17	Ln upper limit	62.96	**upper limit**	n/a
s2	0.13	Ln lower limit	2.87	**lower limit**	17.71

S. aureus 0156 MRSA

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	30.00	1.75	3.40	3.05	xmean
1	30.00	1.25	3.40	1.00	ymean
1/2	15.00	0.75	2.71	0.48	Sxx
1/2	15.00	0.25	2.71		

bo	-3.43	Ln mean	2.37	**mean**	10.67
bi	1.45	Ln upper limit	4.25	**upper limit**	70.15
s2	0.13	Ln lower limit	2.92	**lower limit**	18.63

C. albicans 3153A

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	30.00	1.25	3.40	3.05	xmean
1	30.00	0.75	3.40	0.56	ymean
1/2	15.00	0.25	2.71	0.48	Sxx
1/2	15.00	0.00	2.71		

bo	-3.31	Ln mean	2.61	**mean**	13.60
bi	1.27	Ln upper limit	4.04	**upper limit**	56.76
s2	0.08	Ln lower limit	3.09	**lower limit**	21.92

E.coli 0157:H7 with 100mM NaCl

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	30.00	2.25	3.40	3.05	xmean
1	30.00	1.75	3.40	1.25	ymean
1/2	15.00	0.75	2.71	0.48	Sxx
1/2	15.00	0.25	2.71		

bo	-5.39	Ln mean	2.48	**mean**	11.93
bi	2.17	Ln upper limit	62.96	**upper limit**	n/a
s2	0.13	Ln lower limit	2.87	**lower limit**	17.71

***S. aureus* 1056 MRSA with 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	30.00	0.00	3.40			
1/2	15.00	0.00	2.71			
1/4	7.50	0.00	2.01			
1/8	3.75	0.00	1.32			
bo	n/a	Ln mean	n/a	**mean**		>30
bi	n/a	Ln upper limit	n/a	**upper limit**		n/a
s2	n/a	Ln lower limit	n/a	**lower limit**		n/a

***C. albicans* 3153A with 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	30.00	0.75	3.40		3.05	xmean
1	30.00	0.25	3.40		0.31	ymean
1/2	15.00	0.25	2.71		0.48	Sxx
1/2	15.00	0.00	2.71			
bo	-1.35	Ln mean	2.49	**mean**		12.02
bi	0.54	Ln upper limit	n/a	**upper limit**		n/a
s2	0.08	Ln lower limit	n/a	**lower limit**		n/a

MIC of Pc

***E.coli* 0157:H7**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	125.00	3.00	4.83		3.79	xmean
1/2	62.50	2.50	4.14		2.25	ymean
1/4	31.25	2.00	3.44		2.40	Sxx
1/8	15.63	1.50	2.75			
bo	-0.48	Ln mean	0.67	**mean**		1.95
bi	0.72	Ln upper limit	0.63	**upper limit**		1.87
s2	0.00	Ln lower limit	0.71	**lower limit**		2.02

***S. aureus* 0156**
MRSA

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	125.00	2.50	4.83		
1/2	62.50	1.50	4.14		
1/4	31.25	1.00	3.44		
1/8	15.63	0.50	2.75		
bo	-2.18	Ln mean	2.32	**mean**	10.20
bi	0.94	Ln upper limit	0.24	**upper limit**	1.27
s2	0.04	Ln lower limit	2.97	**lower limit**	19.47

C. albicans
3153A

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	125.00	1.75	4.83		4.48	xmean
1/2	125.00	1.25	4.83		1.00	ymean
1/4	62.50	0.75	4.14		0.48	Sxx
1/8	62.50	0.25	4.14			
bo	-5.47	Ln mean	3.79	**mean**		44.29
bi	1.44	Ln upper limit	5.67	**upper limit**		289.15
s2	0.13	Ln lower limit	4.35	**lower limit**		77.61

***E.coli* 0157:H7 with 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	125.00	2.75	4.83		4.48	xmean
1	125.00	2.25	4.83		2.00	ymean
1/2	62.50	1.75	4.14		0.48	Sxx
1/2	62.50	1.25	4.14			
bo	-4.47	Ln mean	3.10	**mean**		22.15
bi	1.44	Ln upper limit	7.07	**upper limit**		1181.57
s2	0.13	Ln lower limit	4.00	**lower limit**		54.56

***S. aureus* 1056 MRSAwith 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc		
1	125.00	0.00	4.83		
1/2	62.50	0.00	4.14		
1/4	31.25	0.00	3.44		
1/8	15.63	0.00	2.75		
bo	n/a	Ln mean	n/a	**mean**	>125
bi	n/a	Ln upper limit	n/a	**upper limit**	n/a
s2	n/a	Ln lower limit	n/a	**lower limit**	n/a

***C. albicans* 3153A with 100mM NaCl**

Dilution	Conc (ug/mL)	Clearing (mm)	Log conc			
1	125.00	2.25	4.83		4.48	xmean
1	125.00	1.75	4.83		1.50	ymean
1/2	62.50	1.25	4.14		0.48	Sxx
1/2	62.50	0.75	4.14			
bo	-4.97	Ln mean	3.44	**mean**		31.32
bi	1.44	Ln upper limit	6.39	**upper limit**		593.20
s2	0.13	Ln lower limit	4.16	**lower limit**		64.12

APPENDIX A2

RAW DATA AND CALCULATIONS FROM MECHANISM OF ACTION STUDIES

A2.1 RAW DATA FROM THE MICRO-BROTH DILUTION MIC METHOD

Organism	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
Escherichia coli O111	1	2	2	16
	1	2	2	16
	2	2	2	8
	2	2	2	8
	3	1	2	16
	3	1	2	16
	4	2	4	16
	4	2	2	16
	5	2	2	8
	5	2	2	16
E. coli UB1005 (rough K-12 strain)	1	0.125	0.25	8
	1	0.125	0.125	8
	2	0.125	0.125	8
	2	0.125	0.125	8
	3	0.125	0.125	8
	3	0.25	0.125	8
	4	0.125	0.125	8
	4	0.125	0.125	8
	5	0.125	0.125	4
	5	0.125	0.125	4
E. coli DC2 (antibiotic-supersusceptible mutant)	1	0.125	0.125	2
	1	0.125	0.125	2
	2	0.125	0.125	2
	2	0.125	0.125	2
	3	0.125	0.125	2
	3	0.125	0.125	2
	4	0.0625	0.0625	1
	4	0.0625	0.125	1
	5	0.125	0.125	2
	5	0.125	0.125	2
E. coli O157:H7	1	2	8	32
	1	2	8	32
	2	2	8	32
	2	2	4	32
	3	2	8	32
	3	2	8	32
	4	2	8	32
	4	2	8	32
	5	2	16	32
	5	2	8	32
Salmonella typhimurium 14028s	1	0.25	0.5	32
	1	0.25	0.5	32
	2	0.25	0.5	32
	2	0.25	0.5	32
	3	0.25	0.5	32
	3	0.25	0.5	32
	4	0.25	0.5	32
	4	0.125	0.25	16
	5	0.25	0.5	32
	5	0.25	0.5	32

Organism	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
Staphylococcus aureus NCTC 4163	1	2	32	64
	1	1	32	64
	2	1	32	64
	2	1	32	64
	3	1	32	64
	3	1	32	64
	4	1	32	64
	4	1	32	64
	5	1	16	64
	5	1	32	64
S. aureus MRSA R147	1	0.5	64	32
	1	0.5	64	32
	2	0.5	64	32
	2	0.5	64	32
	3	0.5	64	64
	3	0.5	64	64
	4	0.5	64	32
	4	0.5	64	32
	5	0.5	64	32
	5	0.5	64	32
S. aureus 1056 MRSA	1	4	16	>64
	1	4	16	>64
	2	4	16	>64
	2	4	16	>64
	3	4	16	>64
	3	4	16	>64
	4	4	16	>64
	4	4	16	>64
	5	4	32	>64
	5	4	32	>64
S. epidermidis clinical isolate	1	0.25	32	32
	1	0.25	16	32
	2	0.25	16	32
	2	0.25	16	32
	3	0.25	16	32
	3	0.25	16	32
	4	0.25	16	32
	4	0.25	16	32
	5	0.5	16	32
	5	0.5	16	32
Enterococcus faecalis ATCC 29212	1	2	32	64
	1	2	16	64
	2	2	32	64
	2	2	32	64
	3	2	32	32
	3	2	32	64
	4	2	32	64
	4	2	64	64
	5	2	32	64
	5	2	32	64

Organism	Run	MIC (μg/mL) SMAP29	OaBac5mini	OaBac7.5mini
S. typhimurium MS4252S (*phoPQ* mutant; defensin supersusceptible)	1	0.125	0.125	2
	1	0.125	0.125	2
	2	0.125	0.125	1
	2	0.125	0.125	2
	3	0.125	0.125	2
	3	0.125	0.125	2
	4	0.125	0.125	2
	4	0.125	0.125	2
	5	0.125	0.125	1
	5	0.0625	0.125	1
Pseudomonas aeruginosa PAO1	1	4	4	16
	1	4	4	16
	2	4	4	16
	2	4	4	16
	3	4	4	32
	3	4	8	32
	4	4	4	16
	4	4	4	16
	5	4	4	16
	5	4	4	16
P. aeruginosa Z61 (antibiotic-supersusceptible mutant)	1	1	8	32
	1	1	8	32
	2	1	4	32
	2	1	8	32
	3	1	8	32
	3	1	8	32
	4	0.5	8	32
	4	1	8	32
	5	1	8	32
	5	1	8	32

Organism	Run	MIC (μg/mL) OaBac7.5mini	OaBac5mini	OaBac7.5mini
Candida albicans 105	1	2	32	64
	1	2	32	64
	2	4	16	64
	2	2	16	64
	3	2	32	64
	3	2	32	64
	4	2	32	64
	4	2	32	64
	5	2	32	64
	5	2	32	64
C. albicans 3153A	1	4	16	>64
	1	4	16	>64
	2	4	16	>64
	2	4	16	>64
	3	4	16	>64
	3	4	32	>64
	4	4	16	>64
	4	4	16	>64
	5	8	32	>64
	5	4	32	>64

A2.2 EXAMPLE CALCULATION OF THE MEAN MIC AND CONFIDENCE INTERVALS FOR THE MEAN FROM THE RAW DATA

Before calculating the mean MIC and confidence intervals the data were log-transformed. This was done to reduce the effect of the outlying data because the data sets were skewed. The mean and standard deviation of the log-transformed data was calculated using the Excel functions "AVERAGE" and "STDEV". The 95% confidence intervals were calculated using the Excel function "CONFIDENCE" where the alpha value was set to 0.05. The limits of the confidence intervals were calculated by adding and subtracting the confidence interval value to the mean. These values for the log-transformed data were then exponentially-transformed to give the mean and confidence interval limits either side of the mean for the untransformed data. The variance on each side of the mean was different due to the log-transformation. The table below shows the calculated figures for the MIC of SMAP29 against *E. coli* O111.

Organism	Run	SMAP29 Ln MIC
E. coli O111	1	0.6931
	1	0.6931
	2	0.6931
	2	0.6931
	3	0.0000
	3	0.0000
	4	0.6931
	4	0.6931
	5	0.6931
	5	0.6931
	mean Ln MIC	0.5545
	standard deviation Ln MIC	0.2923
	confidence interval Ln MIC	0.1811
	upper limit Ln MIC = (mean Ln MIC + confidence interval Ln MIC)	0.7357
	lower limit Ln MIC = (mean Ln MIC - confidence interval Ln MIC)	0.3734
	mean MIC = exp(Ln MIC)	1.74
	upper limit MIC = exp(upper limit Ln MIC)	2.09
	lower limit MIC = exp(lower limit Ln MIC	1.45

A2.3 RAW DATA FROM THE LPS BINDING ASSAY

SMAP29 Run 1

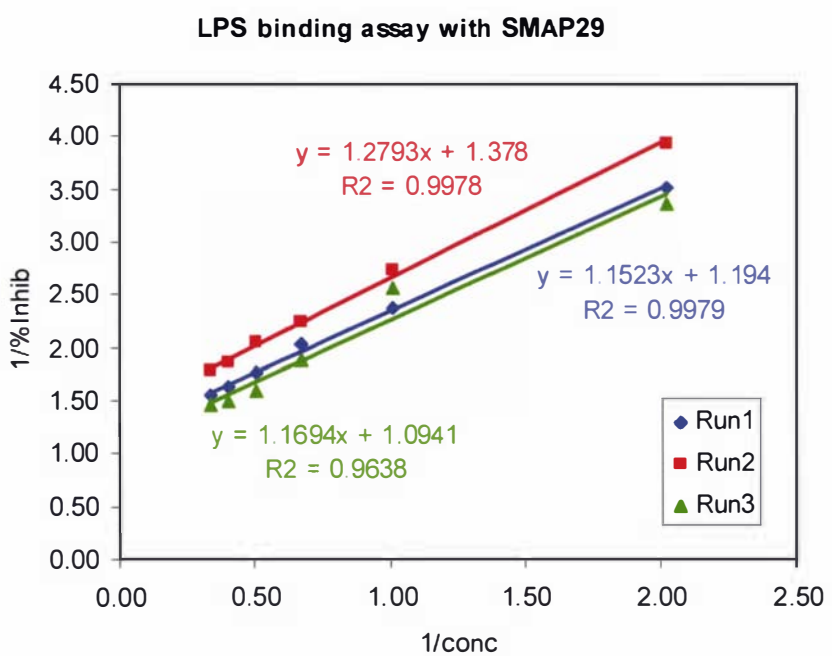
Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		43.8	0.00	
1	0.49	2.02	31.3	0.29	3.50
2	0.99	1.01	25.4	0.42	2.38
3	1.48	0.67	22.2	0.49	2.03
4	1.98	0.51	19.1	0.56	1.77
5	2.47	0.41	17.1	0.61	1.64
6	2.96	0.34	15.5	0.65	1.55

SMAP29 Run 2

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		38.2	0.00	
1	0.49	2.02	28.5	0.25	3.94
2	0.99	1.01	24.2	0.37	2.73
3	1.48	0.67	21.2	0.45	2.25
4	1.98	0.51	19.6	0.49	2.05
5	2.47	0.41	17.6	0.54	1.85
6	2.96	0.34	16.8	0.56	1.79

SMAP29 Run 3

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		29.2	0.00	
1	0.49	2.02	20.5	0.30	3.36
2	0.99	1.01	17.8	0.39	2.56
3	1.48	0.67	13.6	0.53	1.87
4	1.98	0.51	11	0.62	1.60
5	2.47	0.41	9.8	0.66	1.51
6	2.96	0.34	9.2	0.68	1.46



OaBac5mini Run 1

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		41	0.00	#DIV/0!
4	1.98	0.51	34.4	0.16	6.21
8	3.94	0.25	29	0.29	3.42
12	5.91	0.17	27.5	0.33	3.04
16	7.86	0.13	26.6	0.35	2.85
20	9.80	0.10	25.2	0.39	2.59
24	11.74	0.09	24.4	0.40	2.47

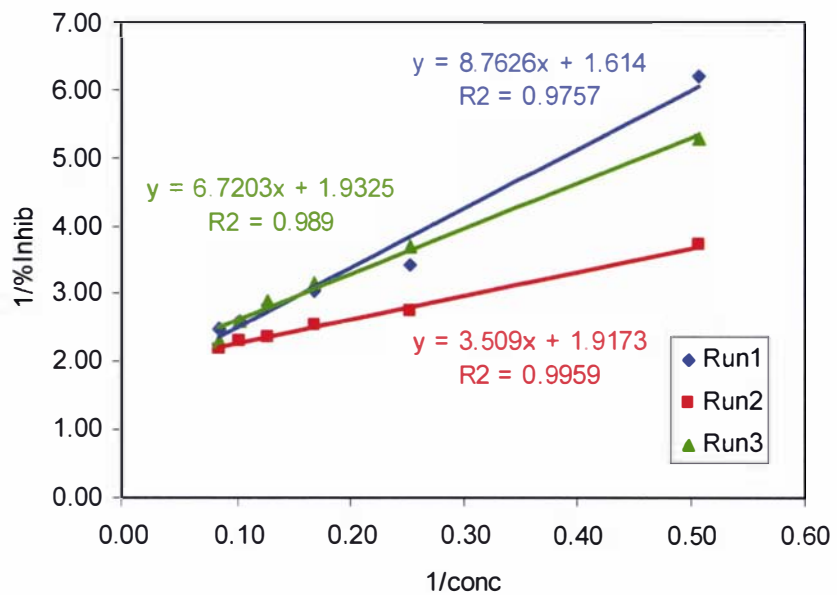
OaBac5mini Run 2

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		43.4	0.00	#DIV/0!
4	1.98	0.51	31.7	0.27	3.71
8	3.94	0.25	27.6	0.36	2.75
12	5.91	0.17	26.3	0.39	2.54
16	7.86	0.13	25.1	0.42	2.37
20	9.80	0.10	24.6	0.43	2.31
24	11.74	0.09	23.6	0.46	2.19

OaBac5mini Run 3

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		35.4	0.00	#DIV/0!
4	1.98	0.51	28.7	0.19	5.28
8	3.94	0.25	25.8	0.27	3.69
12	5.91	0.17	24.2	0.32	3.16
16	7.86	0.13	23.2	0.34	2.90
20	9.80	0.10	21.8	0.38	2.60
24	11.74	0.09	20.1	0.43	2.31

LPS binding assay with OaBac5mini



OaBac7.5 mini Run 1

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		43.4	0.00	
4	1.98	0.51	36.9	0.15	6.68
6	2.96	0.34	34.6	0.20	4.93
8	3.94	0.25	33	0.24	4.17
10	4.93	0.20	31.7	0.27	3.71
12	5.91	0.17	30.6	0.29	3.39
25	12.22	0.08	25.4	0.41	2.41

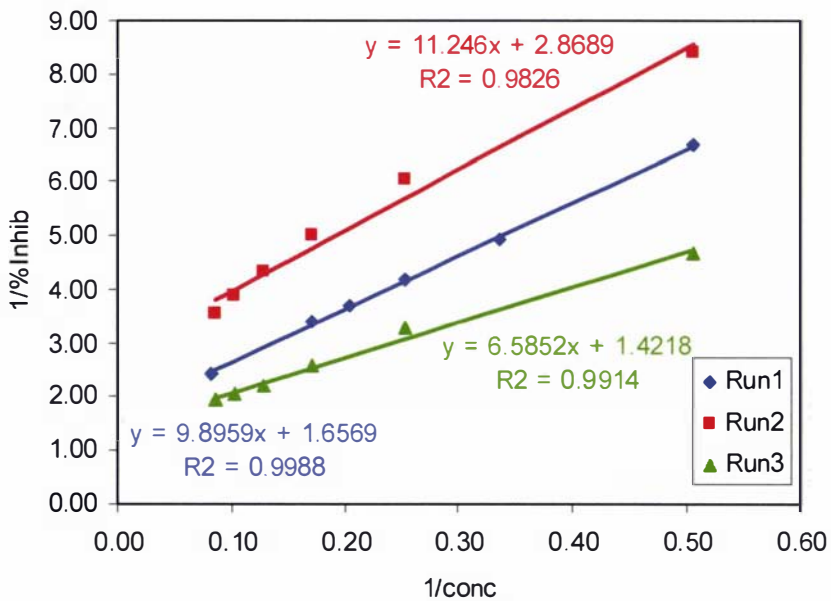
OaBac7.5 mini Run 2

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		36.9	0.00	
4	1.98	0.51	32.5	0.12	8.39
8	3.94	0.25	30.8	0.17	6.05
12	5.91	0.17	29.5	0.20	4.99
16	7.86	0.13	28.4	0.23	4.34
20	9.80	0.10	27.4	0.26	3.88
24	11.74	0.09	26.5	0.28	3.55

OaBac7.5 mini Run 3

Vol added (µl)	Conc (µg/mL)	1/conc	Reading	% Inhib	1/% Inhib
0	0.00		50.1	0.00	
4	1.98	0.51	39.4	0.21	4.68
8	3.94	0.25	34.8	0.31	3.27
12	5.91	0.17	30.6	0.39	2.57
16	7.86	0.13	27.3	0.46	2.20
20	9.80	0.10	25.6	0.49	2.04
24	11.74	0.09	24.4	0.51	1.95

LPS binding assay with OaBac7.5mini



A2.4 CALCULATION OF I_{MAX} AND I_{50} FROM LPS BINDING ASSAY RAW DATA

I_{max} and I_{50} were calculated from the equation of the lines in an Excel worksheet using the following formulas:

I_{max} = the maximum percentage of DPX displaced

$$= \frac{100}{\text{y-intercept}}$$

I_{50} = peptide concentration required to displace half I_{max}

$$= \frac{-1}{\text{x-intercept}}$$

$$\text{x-intercept} = \frac{\text{- y-intercept}}{\text{gradient}}$$

The mean and standard deviation of I_{max} and I_{50} from the three runs were calculated using the Excel functions "AVERAGE" and "STDEV". The results are summarised below.

SMAP29

Run	y-int	gradient	x-int	I_{max}	I_{50}
1	1.19	1.15	-1.04	83.75	0.97
2	1.38	1.28	-1.08	72.57	0.93
3	1.09	1.17	-0.94	91.40	1.07
			mean	**82.57**	**0.99**
			SD	**9.47**	**0.07**

OaBac5mini

Run	y-int	gradient	x-int	I_{max}	I_{50}
1	1.61	8.76	-0.18	61.96	5.43
2	1.92	3.51	-0.55	52.16	1.83
3	1.93	6.72	-0.29	51.75	3.48
			mean	**55.29**	**3.58**
			SD	**5.78**	**1.80**

OaBac7.5mini

Run	y-int	gradient	x-int	I_{max}	I_{50}
1	1.66	9.90	-0.17	60.35	5.97
2	2.87	11.25	-0.26	34.86	3.92
3	1.42	6.59	-0.22	70.33	4.63
			mean	**55.18**	**4.84**
			SD	**18.30**	**1.04**

The probability that there were no differences in the mean I_{max} and I_{50} values from the difference peptides was calculated using Student's t-test. The "TTEST" function in Excel was used. A single tailed t-test for two samples with unequal variances was used. The results are summarised below.

	Imax	**I50**
probability meanSMAP29=meanOaBac5mini	0.0097	0.0651
probability meanSMAP29=meanOaBac7.5mini	0.0523	0.0115
probability meanOaBac5mini=meanOaBac7.5mini	0.4966	0.1831

A2.5 RAW DATA FROM THE OUTER MEMBRANE PERMEABILISATION ASSAY

SMAP29 Run 1

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Uptake
0	0	0.00	0.0	0.0	0.00	0.00
2	0.2	0.20	20.6	30.4	9.80	6.18
4	0.2	0.39	22.7	51.5	28.80	18.15
1	1	0.49	17.1	89.4	72.30	45.56
2	1	0.98	18.5	113.8	95.30	60.05
3	1	1.47	20.1	116.2	96.10	60.55
1	4	1.96	19.0	130.1	111.10	70.01
2	4	3.92	20.0	132.2	112.20	70.70
Polymyxin B standard		1.96	22.6	181.3	158.70	100.00

SMAP29 Run 2

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Uptake
0	0	0.00	0.0	0.0	0.00	0.00
2	0.2	0.20	30.6	55.0	24.40	15.25
4	0.2	0.39	28.3	57.2	28.90	18 06
1	1	0.49	50.6	119.8	69.20	43.25
2	1	0.98	45.0	156.2	111.20	69.50
3	1	1.47	56.4	140.2	83.80	52.38
1	4	1.96	46.6	164.2	117.60	73.50
2	4	3.92	56.4	184.0	127.60	79 75
Polymyxin B standard		1.96	20.0	180.0	160.00	100.00

SMAP29 Run 3

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Uptake
0	0	0.00	0.0	0.0	0.00	0.00
2	0.2	0.20	16.8	24.7	7.90	4.39
4	0.2	0.39	22.4	46.7	24.30	13.50
1	1	0.49	19.2	109.9	90.70	50.39
2	1	0.98	39.2	161.9	122.70	68.17
3	1	1.47	35.4	153.3	117.90	65.50
1	4	1.96	39.3	179.2	139.90	77.72
2	4	3.92	36.8	194.1	157.30	87.39
Polymyxin B standard		1.96	37.3	217.3	180.00	100.00

OaBac5mini Run 1

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	1	0.00	0.0	0.0	0.0	0.00
2	0.2	0.20	29.6	46.4	16.8	6.33
4	0.2	0.39	34.3	61.1	26.8	10.09
1	1	0.49	25.9	120.9	95.0	35.77
2	1	0.98	37.4	272.5	235.1	88.52
3	1	1.47	34.8	244.8	210.0	79.07
1	4	1.96	27.7	240.5	212.8	80.12
2	4	3.92	39.3	277.0	237.7	89.50
Polymyxin B standard		1.96	41.7	307.3	265.6	100.00

OaBac5mini Run 2

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	1	0.00	0.0	0.0	0.0	0 00
2	0.2	0.20	40.8	50.7	9.9	3.74
3	0.2	0.29	42.9	63.4	20.5	7 75
1	1	0.49	47.1	168.6	121.5	45.92
2	1	0.98	47.1	231.5	184.4	69.69
3	1	1.47	42.5	277.9	235.4	88.96
4	1	1.96	47.6	275.4	227.8	86.09
8	1	3.91	47.4	294 8	247.4	93.50
Polymyxin B standard		1.96	37.5	302.1	264.6	100.00

OaBac5mini Run 3

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	1	0.00	0.0	0.0	0.0	0.00
2	0.2	0.20	40.6	50.0	9.4	4.18
3	0.2	0.29	45.7	78.9	33.2	14.76
1	1	0.49	44.4	208.3	163.9	72.84
2	1	0.98	42.0	195.1	153.1	68.04
3	1	1.47	33.1	228.4	195.3	86.80
4	1	1.96	42.1	243.9	201.8	89.69
8	1	3.91	41.1	231.5	190.4	84.62
Polymyxin B standard		1.96	35.0	260.0	225.0	100.00

OaBac7.5mini Run 1 (16/7/02)

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	0	0.00	0.0	0.0	0.0	0.00
2	0.2	0.20	23.1	30.5	7.4	4.40
3	0.2	0.29	19.8	30.2	10.4	6.18
1	1	0.49	20.4	97.0	76.6	45.54
2	1	0.98	24.2	134.8	110.6	65.76
3	1	1.47	25.9	156.2	130.3	77.47
1	4	1.96	31.2	210.0	178.8	106.30
2	4	3.92	32.4	200.0	167.6	99.64
Polymyxin B standard		1.96	41.8	210.0	168.2	100.00

OaBac7.5mini Run 2 (31/7/02)

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	0	0.00	0.0	0.0	0.0	0.00
2	0.2	0.20	32.0	37.2	5.2	2.46
3	0.2	0.29	33.9	47.4	13.5	6.40
1	1	0.49	40.3	139.6	99.3	47.04
2	1	0.98	45.8	201.8	156.0	73.90
3	1	1.47	44.4	227.0	182.6	86.50
1	4	1.96	46.2	279.0	232.8	110.28
2	4	3.92	49.8	265.3	215.5	102.08
Polymyxin B standard		1.96	29.5	240.6	211.1	100.00

OaBac7.5mini Run 3 (5/8/02)

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Intitial	Sample	Increase	% Uptake
0	1	0.00	0.0	0.0	0.0	0.00
2	0.2	0.20	41.7	46.2	4.5	3.45
4	0.2	0.39	48.6	57.8	9.2	7.14
1	1	0.49	47.5	107.4	59.9	46.29
2	1	0.98	42.3	132.7	90.4	69.85
3	1	1.47	38.5	144.5	106.0	81.94
4	1	1.96	45.6	185.3	139.7	107.98
8	1	3.91	42.1	171.7	129.6	100.12
Polymyxin B standard		1.96	44.5	173.9	129.4	100.00

The % uptake was the % of NPN taken up due to the peptide compared to that taken up due to 4µg/mL polymyxin B. It was calculated using the following formula:

$$\%\ \text{uptake} = \frac{\text{increase due to sample}}{\text{increase due to polymyxin B}} \times 100$$

The mean NPN uptake and the standard deviation for each peptide concentration over the three runs was calculated and graphed using SigmaPlot.

A2.6 ANALYSIS OF VARIANCE OF NPN UPTAKE DATA

The NPN-uptake data from each experiment were analysed to determine if there was a difference between the mean NPN-uptake for the different peptides. This was done using the ANOVA function in the statistical package GenStat. The data was entered into GenStat in 4 columns. The first column contained the peptide concentration, the second column contained the run number, the third column contained the peptide number (1=SMAP29, 2=OaBac5mini, 3=OaBac7.5mini) and the fourth column contained the NPN-uptake. The "general analysis of variance" function was used. The y-variate was "NPN-uptake", the treatment structure was "peptide concentration x peptide" and the blocks was "run". The output below shows the ANOVA results.

```
***** Analysis of variance *****

Variate: NPN_Uptake

Source of variation      d.f.       s.s.       m.s.     v.r.  F pr.

Run_number stratum          2     201.61     100.80     2.30

Run_number.*Units* stratum
Peptide                     2    1029.57     514.78    11.73  <.001
Peptide_concentration       7   89212.61   12744.66   290.41  <.001
Peptide.Peptide_concentration
                           14    3132.98     223.78     5.10  <.001
Residual                   46    2018.73      43.89

Total                      71   95595.49
```

A2.7 RAW DATA FROM THE OUTER INNER MEMBRANE DEPOLARISATION ASSAY

SMAP29 Run 1

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	10.2	27.7	17.48	23.24
1	1	0.49	11.5	57.6	46.14	61.36
2	1	0.98	13.2	72.1	58.91	78.34
1	4	1.96	15.5	72.6	57.07	75.89
1.5	4	2.94	11.2	74.0	62.84	83.56
2	4	3.92	18.9	83.4	64.47	85.73
Gramicidin S standard		1.96	14.3	89.5	75.20	100.00

SMAP29 Run 2

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	9.6	28.6	18.99	25.56
1	1	0.49	8.3	67.6	59.28	79.78
2	1	0.98	5.6	71.7	66.07	88.92
1	4	1.96	11.3	63.2	51.93	69.89
1.5	4	2.94	14.2	71.4	57.15	76.92
2	4	3.92	15.1	77.9	62.77	84.48
Gramicidin S standard		1.96	8.7	83.0	74.30	100.00

SMAP29 Run 3

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	14.2	34.5	20.31	21.45
1	1	0.49	15.6	77.9	62.28	65.77
2	1	0.98	16.8	90.3	73.53	77.65
1	4	1.96	15.2	88.0	72.80	76.87
1.5	4	2.94	17.9	89.5	71.64	75.65
2	4	3.92	18.3	94.6	76.27	80.54
Gramicidin S standard		1.96	12.2	106.9	94.70	100.00

OaBac5mini Run 1

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	15.1	24.4	9.28	12.34
1	1	0.49	16.3	27.4	11.10	14.76
2	1	0.98	15.9	34.6	18.70	24.87
1	4	1.96	17.2	31.7	14.48	19.25
1.5	4	2.94	18.2	45.0	26.82	35.66
2	4	3.92	16.3	45.8	29.52	39.25
Gramicidin S standard		1.96	14.3	89.5	75.20	100.00

OaBac5mini Run 2

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	12.2	18.6	6.37	8.58
1	1	0.49	11.5	26.4	14.87	20.01
2	1	0.98	14.3	31.1	16.76	22.56
1	4	1.96	16.8	42.0	25.16	33.86
1.5	4	2.94	19.7	47.8	28.14	37.88
2	4	3.92	20.3	51.7	31.43	42.30
Gramicidin S standard		1.96	8.7	83.0	74.30	100.00

OaBac5mini Run 3

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	16.9	23.2	6.28	6.63
1	1	0.49	17.8	33.1	15.32	16.18
2	1	0.98	20.3	44.7	24.41	25.78
1	4	1.96	21.4	49.3	27.93	29.49
1.5	4	2.94	19.6	56.2	36.60	38.65
2	4	3.92	20.1	57.6	37.48	39.58
Gramicidin S standard		1.96	12.2	106.9	94.70	100.00

OaBac7.5mini Run 1

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	18.5	18.6	0.05	0.07
1	1	0.49	15.4	37.8	22.39	29.78
2	1	0.98	19.5	46.2	26.66	35.45
1	4	1.96	20.4	54.9	34.49	45.86
1.5	4	2.94	21.1	58.9	37.79	50.25
2	4	3.92	20.6	57.2	36.60	48.67
Gramicidin S standard		1.96	14.3	89.5	75.20	100.00

OaBac7.5mini Run 2

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	17.5	17.6	0.10	0.13
1	1	0.49	18.6	45.1	26.51	35.68
2	1	0.98	19.2	43.5	24.27	32.67
1	4	1.96	18.9	61.5	42.60	57.34
1.5	4	2.94	20.5	52.6	32.11	43.21
2	4	3.92	21.6	58.2	36.59	49.25
Gramicidin S standard		1.96	8.7	83.0	74.30	100.00

OaBac7.5mini Run 3

Vol Added (µl)	Conc Added (mg/mL)	Final Conc (µg/mL)	Initial	Sample	Increase	% Released
0	0	0.00	0.0	0.0	0.00	0.00
1	0.2	0.10	19.2	19.2	0.00	0.00
1	1	0.49	20.6	53.5	32.92	34.76
2	1	0.98	21.8	38.7	16.92	17.87
1	4	1.96	21.5	70.2	48.70	51.43
1.5	4	2.94	22.4	71.6	49.23	51.98
2	4	3.92	22.9	74.3	51.42	54.30
Gramicidin S standard		1.96	12.2	106.9	94.70	100.00

The % released was the % of $DiSC_35$ released due to the peptide compared to that released due to 4µg/mL gramicidin S. It was calculated using the following formula:

$$\% \text{ released} = \frac{\text{increase due to sample}}{\text{increase due to gramicidin S}} \text{x} 100$$

The mean $DiSC_35$ released and the standard deviation for each peptide concentration over the three runs was calculated and graphed using SigmaPlot.

A2.8 ANALYSIS OF VARIANCE OF $DiSC_35$ RELEASE DATA

The $DiSC_35$-release data from each experiment were analysed to determine if there was a difference between the mean $DiSC_35$ released for the different peptides. This was done using the ANOVA function in the statistical package GenStat. The data was entered into GenStat in 4 columns. The first column contained the peptide concentration, the second column contained the run number, the third column contained the peptide number (1=SMAP29, 2=OaBac5mini, 3=OaBac7.5mini) and the fourth column contained the $DiSC_35$ released. The "general analysis of variance" function was used. The y-variate was "$DiSC_35$-release", the treatment structure was "peptide concentration x peptide" and the blocks was "run". The output below shows the ANOVA results.

```
***** Analysis of variance *****

Variate: DiSC35

Source of variation      d.f.       s.s.       m.s.     v.r.  F pr.

Run stratum                 2      63.05      31.53     1.62

Run.*Units* stratum
Peptide                     2   15308.66    7654.33   394.25  <.001
peptide_conc                6   27806.03    4634.34   238.70  <.001
Peptide.peptide_conc       12    4925.61     410.47    21.14  <.001
Residual                   40     776.60      19.42

Total                      62   48879.95
```

APPENDIX A3

RAW DATA AND CALCULATIONS FROM EFFECT OF CONDITIONS STUDIES

A3.1 RAW DATA OF MICS AT DIFFERENT SALT CONCENTRATIONS

NaCl conc (mM)	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
0	1	0.5	1	2
0	1	0.5	1	2
0	2	0.5	1	2
0	2	1	1	2
0	3	0.5	1	2
0	3	0.5	1	2
50	1	1	2	8
50	1	1	4	8
50	2	1	4	8
50	2	1	4	8
50	3	1	4	8
50	3	1	4	8
100	1	1	16	32
100	1	1	16	16
100	2	1	16	32
100	2	1	16	32
100	3	2	16	32
100	3	1	16	32
250	1	4	32	64
250	1	2	32	64
250	2	4	32	64
250	2	4	64	64
250	3	4	32	64
250	3	4	32	64

A3.2 RAW DATA OF MICS AT DIFFERENT CATION CONCENTRATIONS

Na+ conc (mM)	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
0	1	0.5	1	2
0	1	0.5	0.5	2
0	2	0.5	1	1
0	2	0.5	1	2
0	3	0.5	1	2
0	3	0.5	1	2
2	1	8	8	8
2	1	4	8	8
2	2	8	8	8
2	2	8	8	8
2	3	8	8	8
2	3	8	8	8
5	1	8	32	8
5	1	8	32	8
5	2	4	32	8
5	2	8	16	8
5	3	8	32	8
5	3	8	32	8
10	1	8	16	16
10	1	4	16	8
10	2	4	32	16
10	2	4	16	16
10	3	4	16	16
10	3	4	16	16

K+ conc (mM)	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
0	1	0.5	1	2
0	1	0.5	0.5	2
0	2	0.5	1	1
0	2	0.5	1	2
0	3	0.5	1	2
0	3	0.5	1	2
2	1	1	16	16
2	1	0.5	16	16
2	2	1	16	8
2	2	1	16	16
2	3	1	16	16
2	3	1	16	16
5	1	4	32	32
5	1	2	16	16
5	2	4	32	32
5	2	4	32	32
5	3	4	32	32
5	3	4	32	32
10	1	4	32	32
10	1	4	16	16
10	2	4	16	16
10	2	4	16	16
10	3	4	16	16
10	3	4	16	16

Mg2+ conc (mM)	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
0	1	0.5	1	2
0	1	0.5	0.5	2
0	2	0.5	1	1
0	2	0.5	1	2
0	3	0.5	1	2
0	3	0.5	1	2
2	1	8	32	64
2	1	8	64	32
2	2	8	64	64
2	2	8	64	64
2	3	8	64	64
2	3	8	64	64
5	1	16	32	32
5	1	32	32	32
5	2	16	64	64
5	2	16	32	32
5	3	16	32	32
5	3	16	32	32
10	1	>64	>64	>64
10	1	>64	>64	>64
10	2	>64	>64	>64
10	2	>64	>64	>64
10	3	>64	>64	>64
10	3	>64	>64	>64

Ca2+ conc (mM)	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
0	1	0.5	1	2
0	1	0.5	0.5	2
0	2	0.5	1	1
0	2	0.5	1	2
0	3	0.5	1	2
0	3	0.5	1	2
2	1	16	64	64
2	1	16	64	64
2	2	16	64	64
2	2	16	32	64
2	3	16	64	64
2	3	16	64	64
5	1	16	>64	>64
5	1	16	>64	>64
5	2	32	>64	>64
5	2	16	>64	>64
5	3	16	>64	>64
5	3	16	>64	>64
10	1	>64	>64	>64
10	1	>64	>64	>64
10	2	>64	>64	>64
10	2	>64	>64	>64
10	3	>64	>64	>64
10	3	>64	>64	>64

A3.3 RAW DATA OF MICS AT DIFFERENT PH VALUES

pH	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
5	1	1	4	8
5	1	1	4	8
5	2	1	4	8
5	2	1	4	8
5	3	1	8	8
5	3	1	4	4
6	1	0.5	8	16
6	1	0.5	8	16
6	2	0.5	8	16
6	2	0.5	8	16
6	3	0.5	8	16
6	3	1	16	16
7	1	1	4	8
7	1	1	2	4
7	2	1	2	4
7	2	1	2	4
7	3	1	2	4
7	3	1	2	4
8	1	0.5	1	2
8	1	0.5	1	2
8	2	0.5	1	2
8	2	1	1	2
8	3	0.5	1	4
8	3	0.5	2	2
9	1	0.5	2	2
9	1	0.5	4	2
9	2	0.5	2	2
9	2	0.5	2	2
9	3	1	2	2
9	3	0.5	2	4

A3.4 RAW DATA OF MICS AFTER HEATING TO DIFFERENT TEMPERATURES

temperature	Run	MIC (μg/mL)		
		SMAP29	OaBac5mini	OaBac7.5mini
not heated	1	0.5	2	2
not heated	1	0.5	1	4
not heated	2	0.5	1	2
not heated	2	0.5	1	2
not heated	3	0.5	1	2
not heated	3	0.5	1	2
30	1	0.5	1	2
30	1	0.5	1	2
30	2	0.5	2	4
30	2	0.5	1	2
30	3	0.5	1	2
30	3	0.5	1	2
40	1	0.5	1	2
40	1	0.5	1	2
40	2	0.5	1	2
40	2	1	1	2
40	3	0.5	1	2
40	3	0.5	1	2
50	1	0.5	2	4
50	1	0.5	1	2
50	2	0.5	1	2
50	2	0.5	1	2
50	3	0.5	1	2
50	3	0.5	1	2
60	1	0.5	2	2
60	1	0.5	1	2
60	2	0.5	1	4
60	2	0.5	1	2
60	3	0.5	1	2
60	3	0.5	1	2
70	1	0.5	1	2
70	1	0.5	1	2
70	2	0.5	1	2
70	2	0.5	1	2
70	3	0.5	1	2
70	3	0.5	1	2
80	1	0.5	2	2
80	1	0.5	1	4
80	2	0.5	1	2
80	2	0.5	1	2
80	3	0.5	1	2
80	3	1	1	2
90	1	4	8	8
90	1	4	8	16
90	2	2	8	8
90	2	4	4	8
90	3	4	8	8
90	3	4	8	8
121	1	4	8	16
121	1	4	8	8
121	2	4	8	8
121	2	4	8	8
121	3	4	8	8
121	3	4	8	8

A3.5 EXAMPLE CALCULATION OF THE MEAN MIC AND CONFIDENCE INTERVALS FOR THE MEAN FROM THE RAW DATA

Before calculating the mean MIC and confidence intervals the data were log-transformed. This was done to reduce the effect of the outlying data because the data sets were skewed. The mean and standard deviation of the log-transformed data was calculated using the Excel functions "AVERAGE" and "STDEV". The 95% confidence intervals were calculated using the Excel function "CONFIDENCE" where the alpha value was set to 0.05. The limits of the confidence intervals were calculated by adding and subtracting the confidence interval value to the mean. These values for the log-transformed data were then exponentially-transformed to give the mean and confidence interval limits either side of the mean for the untransformed data. The variance on each side of the mean was different due to the log-transformation. The table below shows the calculated figures for the MIC of SMAP29 with no salt.

NaCl conc (mM)	Run	SMAP29 Ln MIC
0	1	-0.69315
0	1	-0.69315
0	2	-0.69315
0	2	0
0	3	-0.69315
0	3	-0.69315
	mean Ln MIC	-0.57762
	standard deviation Ln MIC	0.282976
	confidence interval Ln MIC	0.226424
	upper limit Ln MIC = (mean Ln MIC + confidence interval Ln MIC)	-0.3512
	lower limit Ln MIC = (mean Ln MIC - confidence interval Ln MIC)	-0.80405
	mean MIC = exp(Ln MIC)	0.561231
	upper limit MIC = exp(upper limit Ln MIC)	0.703844
	lower limit MIC = exp(lower limit Ln MIC	0.447514

A3.6 ANALYSIS OF VARIANCE OF MIC DATA FROM DIFFERENT CONDITIONS

The MIC data from each experiment were analysed to determine if there was a difference between the mean MICs for the different peptides and for the different conditions. This was done using the ANOVA function in the statistical package GenStat. The data was entered into GenStat in 4 columns. The first column contained the condition (eg NaCl concentration), the second column contained the run number, the third column contained the peptide number (1=SMAP29, 2=OaBac5mini, 3=OaBac7.5mini) and the fourth column contained the Ln MIC. The "general analysis of variance" function was used. The y-variate was "Ln MIC", the treatment structure was "condition x peptide" and the blocks was "run". The output below shows the ANOVA results for the data collected at different salt concentrations.

```
***** Analysis of variance *****

Variate: Ln_MIC

Source of variation      d.f.      s.s.       m.s.     v.r.  F pr.

Run stratum                 2    0.28026    0.14013     3.83

Run.*Units* stratum
Salt_Conc                   3   87.57591   29.19197   797.90  <.001
Peptide                     2   71.62754   35.81377   978.89  <.001
Salt_Conc.Peptide           6   10.15624    1.69271    46.27  <.001
Residual                   58    2.12200    0.03659

Total                      71  171.76195
```

From the output, the F-statistics for the condition and peptide were calculated. For these calculations the interaction error term was used instead of the residual error term. This was done because the residual error was likely to be an underestimate of the variability of the data because the data was discrete, so using the interaction error was a more conservative approach. The calculation of the F-statistics for the salt concentration data are given below.

$$\text{F-statistic}_{\text{peptide}} = \frac{\text{m.s. peptide}}{\text{m.s salt\_conc.peptide}} = \frac{35.81377}{1.69271} = 21.15765$$

$$\text{F-statistic}_{\text{salt conc}} = \frac{\text{m.s. salt\_conc}}{\text{m.s salt\_conc.peptide}} = \frac{29.19197}{1.69271} = 17.24570$$

The F-statistics were used to calculate the probably of the means of each data set being the same. To do this, the Genstat code below was used.

"calc pvalue = CUF(Fstat; d.f.factor;d.f.interaction)"

where Fstat was the calculated F-statistic, d.f.factor was the degrees of freedom of the factor (eg peptide, salt concentration) and d.f.interaction was the degrees of freedom of the interaction term

For the salt concentration data the calculations of the p-values are given below.

Probability that there are no differences between the mean MICs between peptides

= "calc pvalue = CUF(21.15765;2;6)"

= 0.001915

Probability that there are no differences between the mean MICs between salt concs

= "calc pvalue = CUF(17.24570;3;6)"

= 0.002357

The F-statistics and p-values for each set of conditions are summarised in the table below.

Condition	Factor	m.s. factor	d.f. factor	m.s. interaction	d.f. interaction	F-statisitic	p-value
salt conc	peptide	35.81377	2	1.69271	6	21.1577	0.0019
	condition	29.19197	3	1.69271	6	17.2457	0.0024
Na^+ conc	peptide	4.93132	2	1.46138	6	3.3744	0.1042
	condition	25.67754	3	1.46138	6	17.5707	0.0022
K^+ conc	peptide	27.03215	2	1.53256	6	17.6386	0.0031
	condition	27.30352	3	1.53256	6	17.8156	0.0022
Mg^{2+} conc	peptide	6.59288	2	1.50364	6	4.3846	0.0671
	condition	64.42297	3	1.50364	6	42.8447	0.0002
Ca^{2+} conc	peptide	6.45274	2	0.91864	6	7.0242	0.0268
	condition	70.29737	3	0.91864	6	76.5233	0.0000
pH	peptide	32.7349	2	1.5054	8	21.7450	0.0006
	condition	4.5643	4	1.5054	8	3.0320	0.0851
temperature	peptide	23.68752	2	0.13531	16	175.0611	0.0000
	condition	11.07338	8	0.13531	16	81.8371	0.0000

APPENDIX A4

RAW DATA AND CALCULATIONS FROM PILOT-SCALE EXTRACTION STUDIES

A4.1 RAW DATA FROM THE MICRO-BROTH DILUTION MIC METHOD

Organism	MIC (μL crude extract/mL)					
	Run 1		Run 2		Run 3	
Escherichia coli O111	4	4	4	4	4	4
Escherichia coli O157:H7	4	4	8	4	4	4
Salmonella enteritidis	4	4	4	4	4	4
Salmonella typhimurium	8	8	4	8	8	8
Klebsiella pneumoniae	8	4	4	4	4	4
Pseudomonas aeruginosa	4	4	4	4	4	4
Pseudomonas fluorescens	4	4	1	4	4	4
Yersinia enterocolitica	8	4	4	4	4	4
Staphylococcus aureus NCTC 4163	4	4	4	4	4	4
Staphylococcus aureus 1056 MRSA	8	8	16	8	8	8
Streptococcus faecalis	4	4	4	4	4	4
Bacillus cereus	4	8	4	4	4	4
Bacillus nato	4	4	4	4	8	8
Listeria monocytogenes 108 A	2	2	2	2	2	2
Listeria monocytogenes NCTC 10884	2	2	4	2	2	2
Listeria monocytogenes NCTC 7973	4	4	4	4	4	4
Candida albicans 3153A	32	32	32	32	32	32

A4.2 EXAMPLE CALCULATION OF THE MEAN MIC AND CONFIDENCE INTERVALS FOR THE MEAN FROM THE RAW DATA

Before calculating the mean MIC and confidence intervals the data were log-transformed. This was done to reduce the effect of the outlying data because the data sets were skewed. The mean and standard deviation of the log-transformed data was calculated using the Excel functions "AVERAGE" and "STDEV". The 95% confidence intervals were calculated using the Excel function "CONFIDENCE" where the alpha value was set to 0.05. The limits of the confidence intervals were calculated by adding and subtracting the confidence interval value to the mean. These values for the log-transformed data were then exponentially-transformed to give the mean and confidence interval limits either side of the mean for the untransformed data. The variance on each side of the mean was different due to the log-transformation. The table below shows the calculated figures for the MIC of the crude extract against *E. coli* O157:H7.

Organism	Run	Crude extract Ln MIC
E. coli O157:H7	1	1.39
	1	1.39
	2	2.08
	2	1.39
	3	1.39
	3	1.39
	mean Ln MIC	1.50
	standard deviation Ln MIC	0.28
	confidence interval Ln MIC	0.23
	upper limit Ln MIC = (mean Ln MIC + confidence interval Ln MIC)	1.73
	lower limit Ln MIC = (mean Ln MIC - confidence interval Ln MIC)	1.28
	mean MIC = exp(Ln MIC)	4.5
	upper limit MIC = exp(upper limit Ln MIC)	3.6
	lower limit MIC = exp(lower limit Ln MIC	5.6

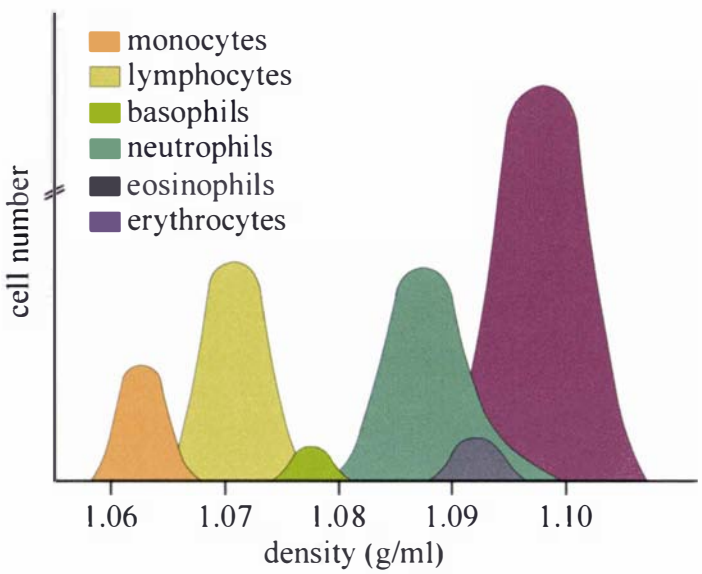
A4.3 CALCULATION OF THE SETTLING VELOCITIES OF DIFFERENT TYPES OF BLOOD CELLS

To determine whether it is possible to separate the white blood cells from the red blood cells using a centrifugation step only, data about the various blood cell types were required. The table below shows the sizes, and the figure below shows the densities of the different cell types.

Blood cell sizes and distribution of various types of white blood cells.

Cell type	Cell diameter (μm)	Portion of total leukocytes (%)
monocytes	12-19	3-8
lymphocytes	8-12	20-25
basophils	7-10	0.5-1
neutrophils	10-12	60-70
eosinophils	12-15	2-4
erythrocytes	6-8	n/a
platelets	2-4	n/a

These data were taken from the websites of the Department of Anatomy and cell biology, Indiana University School of Medicine (http://anatomy.iupui.edu/courses/histo_D502/D502f03/f03_labs/Lab7/Lab7f03.html) and the Wadswroth Centre Clinical Chemistry and Haemotology (New York State Department of Health) (http://www.wadsworth.org/chemheme/heme/microscope/seg.htm).



Graph showing the density distribution and relative amounts of the different cells present in blood. This image was adapted from that given in the OptiPrep catalogue (www.shiyaku-daiichi.jp/catalog-pub/shiyaku/optiprep/C09.pdf).

To determine the settling velocity of the solids in a centrifuge the equation below was used (Meat and Livestock Australia, 2000).

$$V_s = \frac{G\,(\rho_S - \rho_L)\,d^2}{18\mu}$$

Where V_s is the settling velocity of the solid (m/s), G is the acceleration due to the centrifugal gravity (m/s^2), ρ_S is the density of the solid (g/mL), ρ_L is the density of the liquid (g/mL), d is the diameter of the solid particles (mm) and μ is the viscosity of the liquid (Pa.s).

In the case of separation of the different blood cells from the blood plasma, G and μ are constant. This means that the settling velocities of the various cell types are dependent on their densities and their diameters according to the equation below.

$$V_s \propto (\rho_S - \rho_L)\,d^2$$

Where V_s is the settling velocity of the solid (m/s), ρ_S is the density of the solid (g/mL), ρ_L is the density of the liquid (g/mL) and d is the diameter of the solid particles (mm).

e.g. For a neutrophil of average size and density:

$$V_s \propto (\rho_S - \rho_L)\,d^2$$
$$\propto (1.088 - 1)\,11^2$$
$$\propto 10.6$$

This value was calculated for the average density as well as the minimum and maximum for each type of blood cell. The results are summarised in the table below.

Comparison of the component proportional to the settling velocities inside a centrifuge for different blood cell types.

Cell type	Minimum $(\rho_S-\rho_L)d^2$	Average $(\rho_S-\rho_L)d^2$	Maximum $(\rho_S-\rho_L)d^2$
monocytes	6.3	27.2	61.2
lymphocytes	2.6	5.6	7.7
basophils	7.8	12.7	18.2
neutrophils	7.2	15.5	25.3
eosinophils	7.5	13.8	21.6
erythrocytes	3.5	5.2	6.8

Since the densities and sizes of the various types of blood cells are different, their settling velocities due to centrifugal gravity are also different. Although the red blood cells are the densest they are also the smallest of the blood cells, which means that their settling velocity

would be slower than most of the other cell types. Of the white blood cells, lymphocytes have a settling velocity range similar to that of the red blood cells. This means that it would not be possible to separate these two types of cells by centrifugation. However the white blood cells of interest, the neutrophils, have a settling velocity range that is higher than that of the red blood cells.

APPENDIX A5

PEER-REVIEWED PUBLICATIONS

A5.1 OVINE ANTIMICROBIAL PEPTIDES: NEW PRODUCTS FROM AN AGE-OLD INDUSTRY

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.