ISOLATION AND CHARACTERISATION OF HOST DEFENCE PEPTIDES OSTRICACINS FROM OSTRICH HETEROPHILS

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ABSTRACT

Host defence peptides are ubiquitous components of innate immunity within all living organisms. These peptides are small, positively charged and amphiphilic molecules. The biological roles of these peptides are direct antimicrobial activity against pathogens and to induce the innate and adaptive immune response within the host.

The research presented in this thesis was focused on isolating host defence peptides from ostrich blood and characterising their antimicrobial properties. Four ostrich β-defensins, named ostricacins-1-4 (Osp-1-4), were successfully purified from ostrich blood. These peptides contained 36-42 amino acid residues, with the main residues including: arginines, lysines, glycines and cysteines. The molecular weight of these four ostricacins ranged between 4-5 kDa. They displayed antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria with minimum inhibitory concentration (MIC), ranging between 1-12 μg/ml. In addition, Osp-2 displayed antimicrobial activity against yeast, with MIC of 6.2 μg/ml. Osp-1 and Osp-2 were further characterised with the investigation of the effects of cationic ions and temperature changes on their antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria. The antimicrobial activity of both peptides significantly declined with the presence of cationic ions. Both peptides were relatively stable when heated to temperatures between 30-70°C. Finally, an investigation of the mode of action of Osp-1 and Osp-2 against Gram-negative bacteria was carried out. Both peptides were compared with a sheep cathelicidin, SMAP-29, and a human α-defensin, HNP-1. SMAP-29 showed the strongest affinity to LPS and it was the most potent peptide to cause disruption of the outer and cytoplasmic membrane. The two ostricacins showed stronger affinity than HNP-1 and they also indicated partial permeabilisation of the outer membrane and a slight depolarisation of the cytoplasmic membrane. HNP-1 did not indicate disruption of the outer membranes or depolarisation of the cytoplasmic membrane. Further investigation indicated that the partial disruption allowed the ostricacins to pass through the membranes and interact with the intracellular components. However, these peptides could not inhibit the bacterial colony forming potential, and therefore, they were considered bacteriostatic. It is recommended that further research be carried out to investigate the feasibility of ostricacins in adding value to existing topical products.

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Chapter 1

Literature Review

1.1 Introduction

This opening chapter provides a comprehensive review of related studies in the host defence peptide area, during the last 20 years. It begins with an introduction to the animal and human immune systems, which can be divided into two major responses: innate and adaptive immunity. Antimicrobial peptides, which are the major part of this review, are part of the immune system that can be found in granules of neutrophils in blood or epithelial cells of tissues. In addition to antimicrobial properties, they induce two types of immune responses within living organisms. The modes of actions of antimicrobial peptides are known to work on the bacterial membrane as well as the intracellular components. Defensins, one of the major antimicrobial peptides families, can be isolated from vertebrates, insects and plants. In vertebrates, they exist in three different forms: α, β and θ-defensins. This review describes the biological roles of defensins in the immune system. In avian blood, the defence mechanism relies on the non-oxidative mechanism, as the granulocytes lack peroxidase and alkaline phosphatase. Therefore, the microbicidal activity of avian blood is more likely to depend on lysozyme and the cationic antimicrobial peptides. To date, antimicrobial peptides from avian species, which have been successfully purified from chicken, turkey, ostrich and king penguin, were mainly composed of β-defensins. They can be classified into heterophil and non-heterophil. Techniques used to purify and characterise the avian β-defensins will be briefly described. The avian antimicrobial peptides have a broad range of antimicrobial spectrum. Evolutionary relationships to other β-defensins will show that they were related to other mammalian β-defensins. This chapter closes with a discussion concerning the current development of antimicrobial peptides in therapeutic industries and other potential applications for ostrich antimicrobial peptides.

1.2 Animal and Human Immune Systems

Animals and humans face constant assault by invading microorganisms, which are usually pathogenic and harmful, through direct contact, ingestion and inhalation. These pathogenic microorganisms can be in the form of bacteria, viruses, fungi or protozoans. Thus, animals and humans have developed natural defence mechanisms that enable them to prevent and eliminate infections caused by pathogens. This defence system can be divided into external barriers, comprised of an intact layer of epithelial skin and mucous tissue membranes, which provide physical and chemical protection against the entry of microorganisms, and any microbes that succeed in breaching the physical barriers (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Playfair and Bancroft, 2004).

The immune system is a complex network of organs, cells and molecules scattered throughout the body, that mediate resistance to infection caused by pathogens. It has a recognition system to identify the presence of invading microbes, a disposal system that kills and eliminates the invading microbes and a communication system that coordinates the activities of the various recognition and disposal systems. There are two types of responses: innate and adaptive immunity (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Girardrin and Philpott, 2004),(Playfair and Bancroft, 2004),(Izadpanah and Gallo, 2005),(Tossi, 2005). Innate immunity is always present in healthy individuals and it prepares to mediate and rapidly eliminate any invading microbes by using phagocytic cells. This response does not require prior exposure to foreign pathogens. Adaptive immunity is activated by stimulation from the invading microbes, which manage to shake off the innate immunity. Adaptive immunity is a relatively more specific immune response than innate immunity. It also develops more slowly. A summary of the diversity of each immune response is illustrated in Table 1.1. Despite the fact that the two responses are diverse, both are involved in a complex interaction to eliminate infections caused by pathogens.

Table 1.1 Comparison of Innate and Adaptive Immune Response adapted from (Playfair and Bancroft, 2004).

	Innate Immunity	Adaptive Immunity
Evolutionary origin	Invertebrates and vertebrates	Only vertebrates
Principal cells	Phagocytic cells	Lymphocytes
Principal molecules	Complement system and cytokines	Antibody and cytokines
Recognition specificity	Broad	High specificity
Speed of action	Rapid (minutes to hours)	Slow (days)
Development of memory	No	Yes

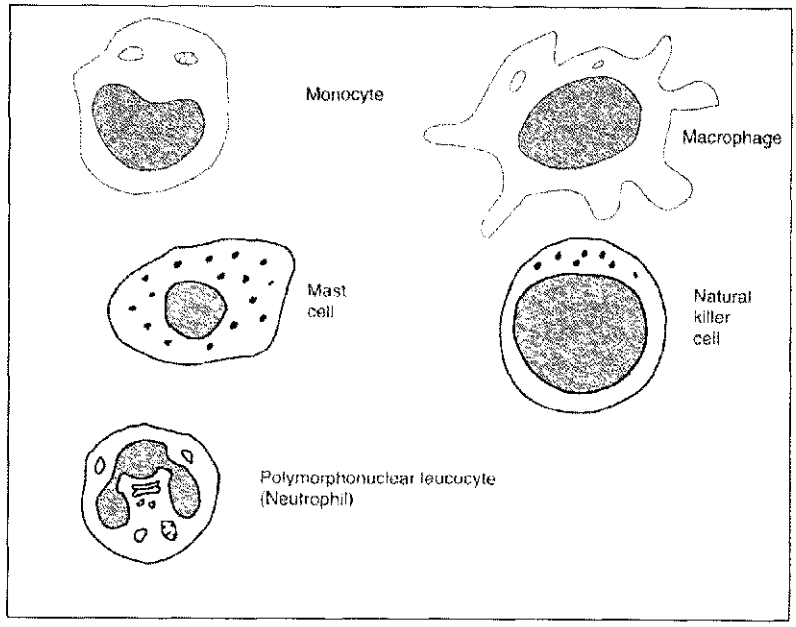
1.2.1 Innate Immunity

Innate immunity is also known as natural, native and non-specific immunity. Innate immunity can be found in all animals (vertebrates and invertebrates) as it has been conserved throughout evolution. The innate immunity components, illustrated in Figure 1.1, include neutrophil granulocytes, monocytes/macrophages, mast cells, natural killer (NK) cells, a complement cascade system and also antimicrobial substances, such as lysozyme, antimicrobial proteins and antimicrobial peptides that act readily against the invading pathogens (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Playfair and Bancroft, 2004).

The main principal component of the innate immunity system is phagocytic cells (phagocytes). They are found abundantly in the blood, as they are part of the white blood cells (leukocytes). There are two types of phagocytes: polymorphonuclear leukocytes (PMN), also known as neutrophils, and monocytes/macrophages. Neutrophils are the most abundant leukocytes in the blood (60-70% of leukocytes). In response to infection, the production of neutrophils from bone marrow increases rapidly with cytokines stimulations. These cells are highly phagocytic, motile and very active in the initial stages of infection. They have the ability to leave blood and rapidly enter the infected extravascular tissues, where they destroy the microbes or foreign particles. Monocytes, which are less abundant than neutrophils (3-8% of leukocytes), are the precursor of macrophages. They are not actively phagocytic until they leave the blood, enter body tissues and differentiate into macrophages. Migration of these cells to the

infection site is also triggered by cytokines. Some macrophages reside locally in certain tissues and organs of the body, such as the liver, lungs, nervous system, bronchial tubes, spleen, lymph nodes, bone marrow and the peritoneal cavity of abdominal organs.

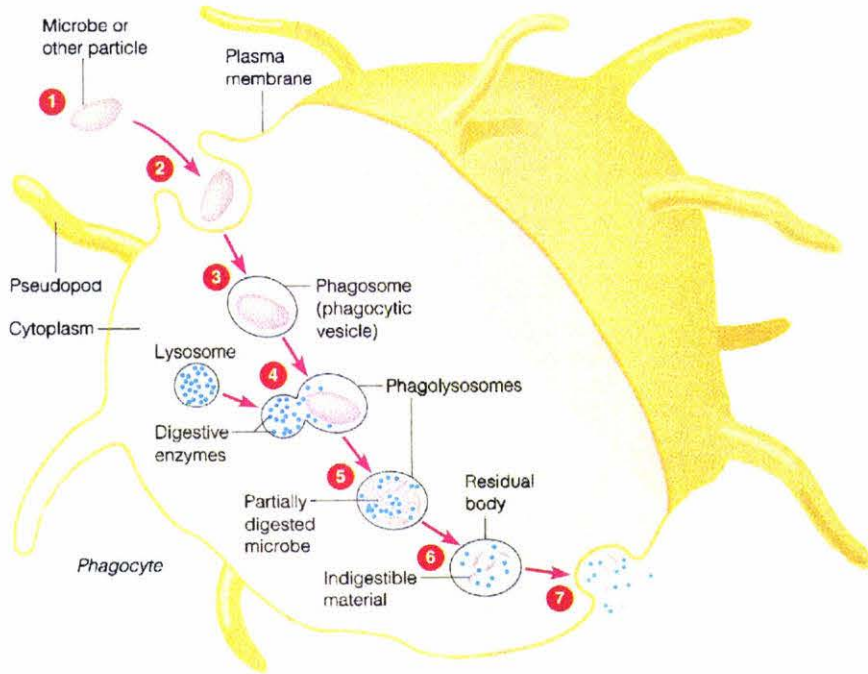
Figure 1.1 Molecular Components of Innate Immune System adapted from (Playfair and Bancroft, 2004).



The hosts are able to recognise microorganisms in the blood or extravascular tissues through pattern-recognition molecules, which include a complement cascade system and a Toll-like receptor (TLRs) family (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Girardrin and Philpott, 2004),(Playfair and Bancroft, 2004),(Izadpanah and Gallo, 2005),(Tossi, 2005). These receptors are homologous to Toll receptors, which are membrane-anchored proteins, involved in innate immune defence against Gram-positive bacteria and fungal infection in the fruit fly *Drosophila*. TLRs are capable of recognising pathogen-associated molecular patterns (PAMP), which are specific microbial components, located in the surface of microorganisms, such as lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acid (LTA) of Gram-positive, bacterial peptidoglycan, cell wall components of yeast and fungi and double-stranded and single-stranded RNA of viruses. Upon recognition of the PAMPs, the TLRs generate signals that stimulate production of cytokines, which lead to recruitment and maturation of phagocytes. Once matured, the phagocytes inactivate the microorganisms in a process known as phagocytosis. Firstly, the phagocyte extends its

plasma membrane around and encapsulates them in a membrane-bound vesicle called phagosome. The phagosome then fuses with lysosomes to form phagolysosomes, where the microbes are digested with various antimicrobial substances, such as microbicidal enzyme, antimicrobial protein and peptides. An illustration of the phagocytosis process is shown in Figure 1.2.

Figure 1.2 The Phagocytosis Mechanisms of a Phagocyte on a Microbe or a Foreign Particle adapted from (Tortora, *et al.*, 1998).



Another important component of innate immunity is mast cells. Mast cells can be found extensively in the skin, around blood vessels, in the gut and in basophils cells of blood. They are designed to release the contents of their granules, such as histamine, which increase vascular permeability and the supply of blood and its immunological components (PMNs, monocytes/macrophages, antibody, etc). They play a role in the acute inflammatory response at the sites of infection. Natural killer (NK) cells are another well-known innate immunity component. NK cells are actually a class of lymphocytes (comprising 10% of blood and peripheral lymphoid organs) that kill infected or cancerous cells and produce interferon-γ (IFN-γ). NK cells are less restricted in their recognition and they respond more rapidly than adaptive T cells. The key feature of NK cells is that they can distinguish infected or cancerous cells from normal

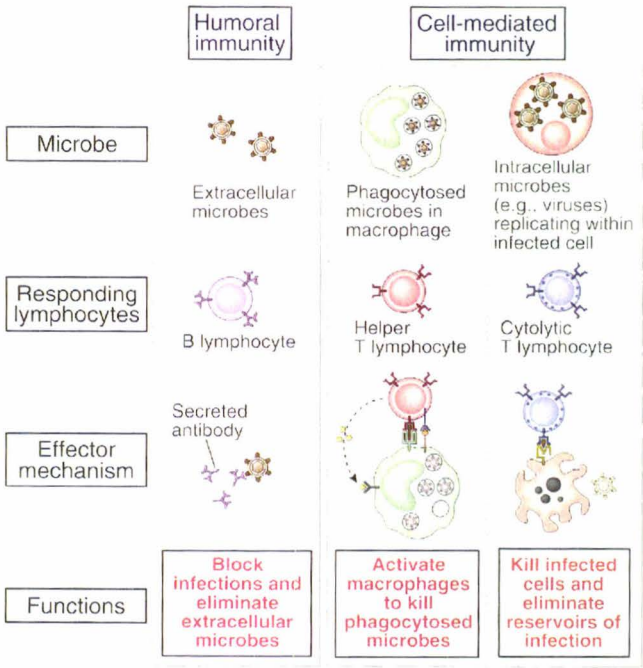
cells through expressions of various receptors that recognise cell surface molecules of infected/cancerous cells. Another key feature of NK cells is the production of IFN-γ, which in turn activates macrophages to kill phagocytosed pathogens more effectively.

1.2.2 Adaptive Immunity

Adaptive immunity is also known as specific or acquired immunity because it allows individuals to make their own set of recognition molecules based on microorganisms they have encountered (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Playfair and Bancroft, 2004). Adaptive immunity is only found in higher animals, such as vertebrates. This immune response may be induced actively, through vaccination (artificial) or through infection to specific microorganisms or foreign molecules (natural). It can also be stimulated passively, through transfer from active immunised individuals (natural) or through the introduction of antibodies from individuals who have already developed an immune response to a particular disease (artificial).

The main components of adaptive immunity are lymphocytes that express receptors, which specifically recognise different substances of antigens. Antigens can be microorganisms or foreign molecules. Lymphocytes and their products are designed to mediate and exclude extracellular and intracellular microbes. Based on two types of lymphocytes (B and T-lymphocytes, also known as B and T-cells), adaptive immunity can be divided into humoral immunity and cellular-mediated immunity, as shown in Figure 1.3. Humoral immunity produces B-cells, which in turn produces antibodies that act against specific antigens in bone marrow. With the production of antibodies, B-cells regulate the extracellular body space that includes blood, tissue fluids and other body fluids. Hence, humoral immunity primarily defends against extracellular infection caused by bacteria, bacterial toxin and viruses freely circulating in the body fluid. On the other hand, cellular-mediated immunity produces specialised T-cells in thymus. The primary function of T-cells is to monitor intracellular compartments and to inactivate bacteria or viruses located within phagocytic or infected host cells and tissues. T-cells also greatly enhance antimicrobial mechanisms of innate immunity, including activation and proliferation of macrophage.

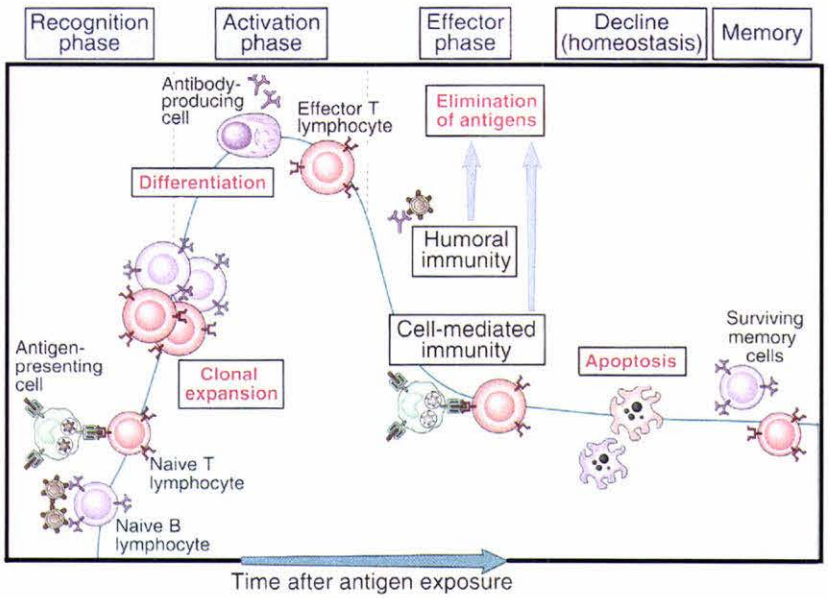
Figure 1.3 Molecular Components of Adaptive Immune System adapted from (Abbas and Lichtman, 2004).



When adaptive immunity recognises microbes, the host responds through sequential phases, as shown in Figure 1.4. In the first exposure to the microbes, the response is known as the primary adaptive immune response. In the first phase (the recognition phase), naïve antigen-specific lymphocytes locate and recognise the microbes. It is called naïve lymphocytes because the cells are not experienced immunologically. The subsequent phase is the activation phase of the lymphocytes that requires two types of signals: signals from the binding of an antigen to an antigen receptor and signals provided by the microbes and the innate immunity responding to the microbes. The second signals are required to ensure that adaptive immunity is elicited by the microbes and not by harmless antigens. During the activation phase, lymphocytes, which have encountered the microbes, undergo clonal expansion, at which time the clones go through rapid cell division to generate a large number of progeny. Some lymphocytes differentiate into effector lymphocytes, which produce a substance for eliminating the microbes (effector phase). The components of innate immunity are often involved in this phase, as a response to the substance produced by the effector lymphocytes. Once the antigen is eliminated, the stimulus of lymphocyte activation is gone and the activated lymphocytes die through a process known as apoptosis, where the dead lymphocytes are eliminated by phagocytes without causing harmful reaction to the host.

The immune response then subsides and the remaining cells, called memory lymphocytes, are able to respond to repeat encounters of the same microbes (secondary immune response). They can survive in a state of rest for months or years. Apart from retaining memory of the microbes, lymphocytes are also highly specific to structurally distinct antigens. Therefore, they are able to distinguish a billion different strains of the same microbes and their components.

Figure 1.4 Adaptive Immune Responses Upon Recognition of Antigens adapted from (Abbas and Lichtman, 2004).



It is believed that the functions of the two lymphocytes complement each other, since the T-cells can recognise many different types of microbial molecules, including proteins, carbohydrates and lipids, whilst the antibodies of B-cells are only specific to microbial protein antigens. The interactions between the two lymphocytes, as well as interactions with the innate immunity, are regulated by a heterogeneous group of mediators known as cytokines (Tortora, *et al.*, 1998),(Abbas and Lichtman, 2004),(Playfair and Bancroft, 2004),(Tossi, 2005). Generally, cytokines are referred to as interleukins, which means they are produced and act on leukocytes. However, more specialised cytokines are available, with the predominant ones called interferons that interfere with virus replication, the tumour necrosis factor (TNF) that causes tumours on mice to shrivel up, the colony-stimulating factor (CSF) that affects the growth of bone marrow cells in culture and the chemokines that stimulate cell migration. Based on these

effects, cytokines can be sorted into five categories, which are those involved in the promotion of: inflammatory responses; cell differentiation and proliferation; cell movement; inhibition; and antiviral.

1.3 Antimicrobial Peptides

Antimicrobial peptides are relatively small cationic molecules comprised of less than one hundred amino acids. They are gene-encoded peptides that have an overall net positive charge, because they contain a number of positive charged residues (histidines, lysines and arginines). In addition, they are composed of at least 50% of hydrophobic residues. The rest of the molecules are comprised of a low proportion of neutral polar and negatively charged amino acids. In solution, these peptides can fold into an amphiphilic structure, where clusters of hydrophobic and cationic amino acids are separated into discrete domains. These properties, small, cationic and amphipathic, are ubiquitous to antimicrobial peptides (Boman, 1991),(Gururaj Rao, 1995),(Hancock and Chapple, 1999),(Mor, 2000),(Hancock, 2001),(Zasloff, 2002),(Boman, 2003),(Brogden *et al.*, 2003),(Kamysz *et al.*, 2003),(Bowdish *et al.*, 2005),(Izadpanah and Gallo, 2005).

Antimicrobial peptides have been known by other names, such as cationic antimicrobial peptides, antibacterial peptides and natural antibiotics. At present, researchers prefer the term 'host defence peptides' because these peptides have demonstrated an ability to stimulate immune responses in addition to their antimicrobial activity (Divine and Hancock, 2004), (Bowdish *et al.*, 2005),(McPhee and Hancock, 2005),(Sahl *et al.*, 2005). In the last 20 years, these peptides have been rapidly used in fundamental research and in the development of new drugs, because of their ability to neutralise microbial infections and to mediate acute and chronic inflammations (Hancock and Chapple, 1999),(Mor, 2000),(Zasloff, 2002),(Kamysz *et al.*, 2003),(Marshall and Arenas, 2003),(Izadpanah and Gallo, 2005). Furthermore, it is believed that they can overcome microbial resistance mechanisms. They are regarded as potential candidates to fight the growing problem of multi drug resistance in bacteria and fungi.

1.3.1 Classes of Host Defence Peptides

Research relating to these endogenous molecules was believed to have begun in the early 20th century, when Dr Elie Metchnikoff won the Nobel prize for describing components involved in phagocytosis (Tizard, 1995),(Tortora *et al.*, 1998). He observed and demonstrated that neutrophils contained some enzymes that enabled the cells to lyse and digest any invading microbes. Thirty years later, Alexander Fleming successfully purified lysozyme, the microbial digestive enzymes described by Metchnikoff. With the development of separation methods of simple and complex chemicals, such as precipitation, crystallisation, filtration, chromatography and electrophoresis, Hussein Zeya and John Spitznagel were able to partially characterise cationic polypeptides purified from rabbits and guinea pigs leukocytes (Spitznagel, 1997). As the biotechnology area developed in the 1970-2000s, more host defence peptides were isolated and characterised using advanced separation and purification techniques (Lehrer, 2004),(Levy, 2004),(Ganz, 2005). There were also host defence peptides characterised from expressions, using cloning techniques of cDNA encoding the peptides.

Over 800 host defence peptides have been isolated and characterised from bacteria, fungi, insects, crustaceans, plants, birds, amphibians, mammals and humans (http://www.bbcm.univ.trieste.it/~tossi/antimic.html) (Andreu and Rivas, 1998),(Brogden *et al.*, 2003),(Kamysz *et al.*, 2003),(Powers and Hancock, 2003),(Marshall and Arenas, 2003),(Reddy *et al.*, 2004),(Zasloff, 2004),(Sahl *et al.*, 2005). The widespread distribution throughout living organisms suggests that these compounds play an essential role as a defence mechanism against pathogenic microorganisms. In animals and humans, they have been isolated from leukocytes and epithelial cells of skin, gastrointestinal and the respiratory tract. Based on structural size and conformational structure, they can be classified into four classes (Figure 1.5) (Martin *et al.*, 1995),(Andreu and Rivas, 1998),(Zasloff, 2002):

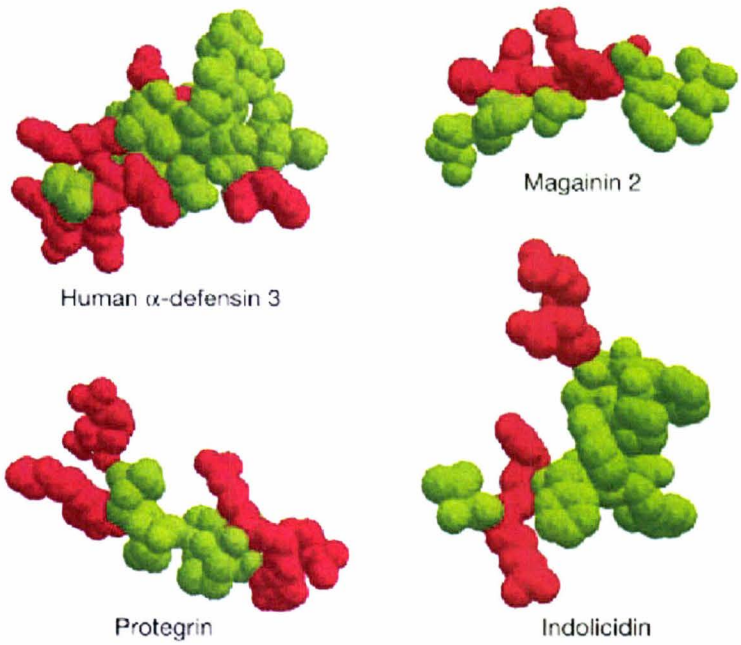
(1) Cysteine-rich amphiphilic β-sheet peptides

These peptides are characterised with the presence of an antiparallel β-sheet, stabilised by disulfide bonds. Defensins are the prominent host defence peptides in this class. They can be found in plants and animals. Some defensins contain helical segments, such as plant and insect defensins.

(2) Amphiphilic α-helical peptides

This class is characterised by a α-helical structure that contains a slight bend in the centre of the molecule. This bend is critical for selectivity by suppressing haemolytic activity. Frog magainins, porcine cecropins, sheep myeloid antimicrobial peptides-29 (SMAP-29) and human LL-37 are well-known amphiphilic α-helical peptides. These peptides are usually unstructured in solution but they form α-helical amphipathic structures in physiological conditions and when they are in contact with biological membranes.

Figure 1.5 Structural Representations of The Four-Classes Host Defence Peptides taken from (Zasloff, 2002).



Human α-defensin 3, a cysteine-rich amphiphilic β-sheet peptides; Magainin 2, an amphiphilic α-helical peptide; indolicidin, a linear peptide with one or two predominant amino acids; protegrin, a cysteine-rich, amphiphilic disulfide ring peptide.

(3) Cysteine-rich, amphiphilic disulfide ring peptides

This class is characterised by a loop structure connected with a single bond, which can be disulphide, amide or isopeptide. Host defence peptides, which are classified in this class, include ranalexin and brevinins isolated from frog skins, protegrins from porcine leukocytes and the classical antibiotic nysin.

(4) Linear peptides with one or two predominant amino acids.

These peptides are generally rich in proline and arginine that represents more than 60% of the overall residues. The secondary structures are not stabilised through inter-residue hydrogen bonds but through hydrogen bonds and Van der Waals interactions with membrane lipids. Bactenecin (Bac-5 and -7) and indolicidin of bovine and PR-39, a proline-rich peptide with 39 amino acids of pig small intestines, are peptides classified in this class.

1.3.2 Host Defence Peptides Roles in Immunity

The primary role of host defence peptides is direct antimicrobial activity to pathogen invasions. These peptides are readily released in response to the presence of microbial products (LPS, LTA, etc.), cell injury and inflammations. In vitro, they have shown an ability to directly kill or inactivate a number of microorganisms, including Gram-negative bacteria, Gram-positive bacteria, fungi, yeast, viruses, parasites and even tumour and malignant cells (Boman, 1991),(Martin *et al.*, 1995),(Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Mor, 2000),(Hancock, 2001),(Brogden *et al.*, 2003),(Kamysz *et al.*, 2003),(Marshall and Arenas, 2003),(Levy, 2004),(Yang and Oppenheim, 2004),(Izadpanah and Gallo, 2005). The antimicrobial activity is limited to the presence of high concentration of salt (NaCl), divalent cations (Ca^{2+} and Mg^{2+}), serum, polyanions and proteases. It is believed that the antimicrobial activity of these peptides is most effective at sites with a low presence of inhibitors, such as in the phagocytic vacuoles and on the surface of skin and mucosal epithelium.

In mammals, host defence peptides have also shown multi-functional roles in innate and adaptive immunity (Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Zasloff, 2002),(Kamysz *et al.*, 2003),(Marshall and Arenas, 2003),(Levy, 2004),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Izadpanah and Gallo, 2005),(Tossi, 2005).

1.3.2.1 Roles in Innate Immunity

The peptides roles in mammalian innate and adaptive immunity are illustrated in Figure 1.6. Firstly, host defence peptides are chemoattractants for innate immunity components (monocytes, macrophages and neutrophils, mast cells) to the sites of microbial entry or

inflammation and they stimulate the local innate immunity. Moreover, host defence peptides enhance phagocytosis by upregulating the expression of adhesion molecules on phagocytes that enhances their recruitment and activation of the phagocytes. This was shown in human α-defensins that could induce the production of reactive oxygen intermediates (Yang and Oppenheim, 2004). Host defence peptides also demonstrate indirect chemotaxis effect by stimulating the production of cytokines. For example, LL-37 and defensins were able to stimulate the production of IL-8 and monocyte chemoattractant protein (MCP-1), which led to the recruitment of more phagocytes to inflammatory sites and the production of TNF-α and IL-1 in monocytes stimulated by the host defence peptides (Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Zasloff, 2002),(Kamysz *et al.*, 2003),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Izadpanah and Gallo, 2005),(Otvos Jr., 2005).

Secondly, host defence peptides can be mediators of inflammations, as they attract and induce degranulation of mast cells at the infection sites in order to release histamine and prostaglandin that lead to vasodilation. Vasodilation enhances migration of phagocytes and other components that help to mediate the infections. Mass cell degranulation was induced by LL-37, magainin-2, defensins and CAP-11 as reported in (Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Izadpanah and Gallo, 2005),(Otvos Jr., 2005). These peptides also stimulated the expression of a variety of genes by macrophages particularly in the production of pro-inflammatory cytokines (IL-8 and MCP-1), which resulted in positive feedback loop that amplified recruitment of the inflammatory cells and subsequently led to degranulation of the phagocytes to release more defensins.

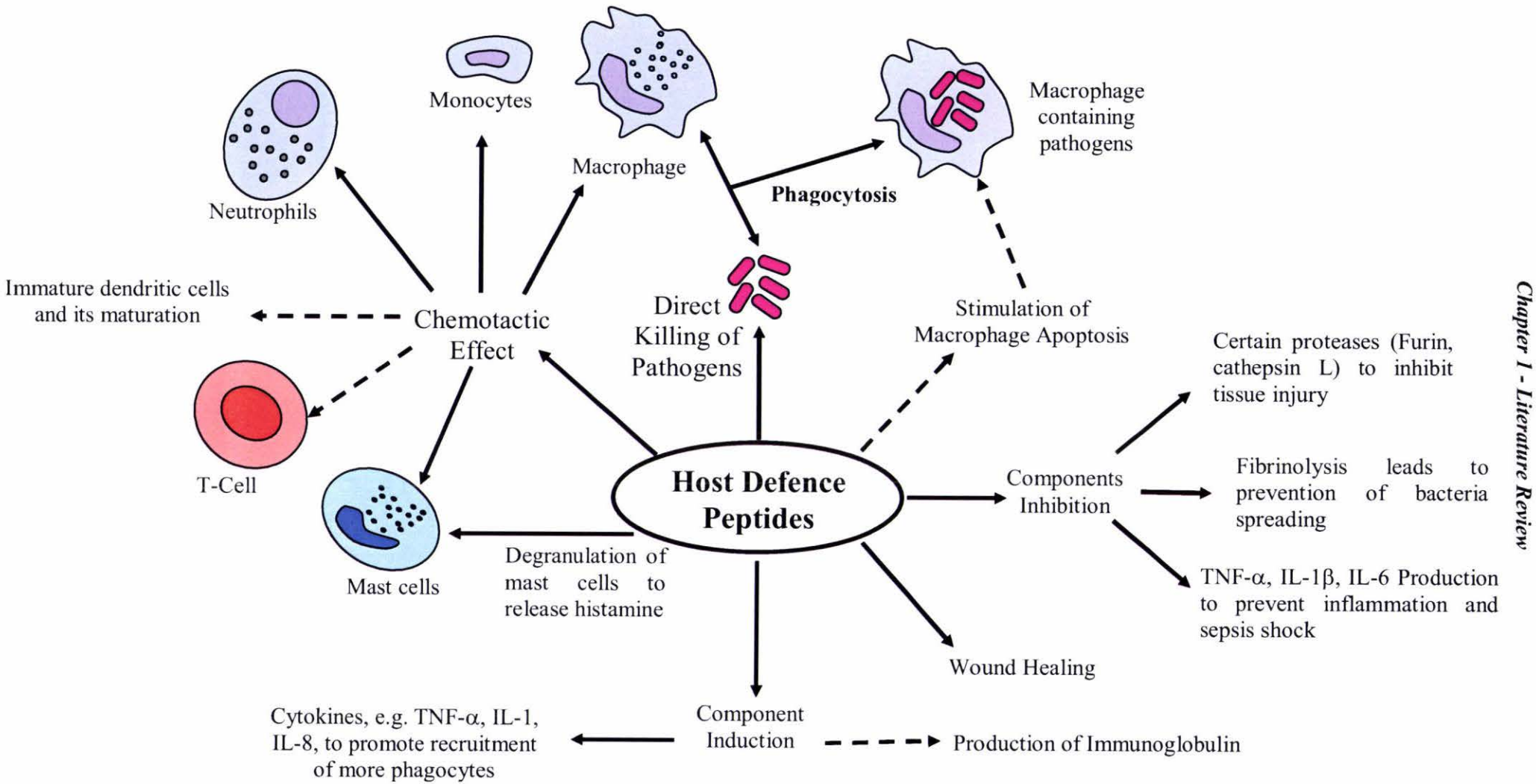


Figure 1.6 Host Defence Peptides Roles in Innate and Adaptive Immunity. This diagram is designed based on previous literature. (→ indicating innate immunity;---> indicating adaptive immunity)

Thirdly, in innate immunity, host defence peptides prevent sepsis shock by the suppression of pro-inflammatory agents (Scott and Hancock, 2000),(Kamysz *et al.*, 2003),(Brogden *et al.*, 2004),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Otvos Jr., 2005). Disintegration of bacteria by phagocytes and host defence peptides causes the release of bacterial components, LPS or LTA, into the circulation system. These components are known to be endotoxic by the fact they trigger high-level production of TNF-α, IL-1β and IL-6 that leads to septic shock and death. These peptides appear to neutralise the endotoxic effect by binding to LPS or LTA that subsequently inhibit the cytokines productions. These peptides can also suppress expression LPS-induced genes in macrophages that reduce the production of TNF-α, IL-1β and IL-6. Hancock *et al* have reported peptides such as LL-37, SMAP-29, indolicidin, human α-defensins and lactoferrin neutralised the endotoxic effect. However, this role is contrary to that formerly described (production of IL-8 and MCP-1). One possible explanation is that the innate immunity is designed to contend with the infection effects by suppression of pro-inflammatory cytokines to limit the induction of septic levels, as well as permitting other cytokines involved in mediation of inflammations.

Lastly, host defence peptides, as shown by defensins and CAP-37, promote wound healing through epithelial cell proliferation and stimulation of fibroblast growth (Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Kamysz *et al.*, 2003),(Bowdish *et al.*, 2005),(Otvos Jr., 2005). The rate of wound healing was amplified to prevent an invasive opportunity for many other pathogens to infect the host. LL-37 was another peptide reported to promote wound healing via angiogenesis stimulation, a process for healing wounds and restoring blood flow to tissues after injury.

1.3.2.2 Roles in Adaptive Immunity

The role host defence peptides in adaptive immunity was chemoattractants for T-cells (Hancock and Diamond, 2000),(Scott and Hancock, 2000),(Zasloff, 2002),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Otvos Jr., 2005). As described above, the peptides chemotactic ability could amplify innate immunity, which consequently led to recruitment of more adaptive immunity components to the infection/inflammation sites,

guided by traditional chemokine receptors (e.g. IL-8, MCP-1). Some peptides that showed chemotactic ability, which included human α-defensins 1-3 that were chemotactic for CD45RA, naïve $CD4^+$ T Cells and $CD8^+$ T Cells and LL-37 that were chemotactic for peripheral T cell to sites of infection.

Host defence peptides are also chemoattractant to immature dendritic cells. Dendritic cells exist in two types: immature (iDC) and mature dendritic cells (mDC). Immature dendritic cells have a phagocytic ability and produce an array of mediators, including cytokines, chemokines and host defence peptides. These cells are derived from circulating haematopoietic precursor cells and pre-dendritic cells populations (monocytes and plasmacytoid cells). Human β-defensin 2 has shown chemotactic ability for iDC and memory or effector T-cells through interaction with chemokine receptor CCR-6 (Scott and Hancock, 2000),(Zasloff, 2002),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005),(Otvos Jr., 2005). The interaction of β-defensins with CCR-6 led to migration of iDC from blood to the skin and from inflammation sites to local lymph nodes, with subsequent activation of memory specific T-cells. Host defence peptides also demonstrate ability to directly and indirectly stimulate maturation of dendritic cells. Maturation of dendritic cells usually takes place after antigen has been taken up and identified by iDCs. Mature dendritic cells then become effective at antigen-processing and presenting the mDCs via expression of a chemokine receptor, CCR-7, which enable them to migrate to regional lymph nodes and interact with naïve T-cells. Mature dendritic cells also determined the nature and consequences of the interaction that cause proliferation and differentiation or deletion of T-cells. Mouse β-defensin 2 was able to directly induce maturation of DC, as demonstrated by the upregulation of CD86, CCR-7 and the major histocompatibility complex (MHC) class II (Yang and Oppenheim, 2004), whilst human α-defensins indirectly stimulated maturation of iDC by inducing the production of TNF-α and IL-1β (Bowdish *et al.*, 2005).

Furthermore, host defence peptides are capable of inducing antigen-specific immune responses in vivo (production of immunoglobulin IgG) (Hancock and Diamond, 2000),(Yang and Oppenheim, 2004),(Bowdish *et al.*, 2005). The responses were demonstrated by simultaneous intranasal administration of ovalbumin (OVA) and a mixture of human α-defensins 1-3 that enhanced production of OVA-specific serum IgG antibody and ex vivo generation of IFNγ, IL-5, IL-6 and IL-10 by OVA-specific

$CD4^+$ T-cells. In addition, mice treated with an intraperitoneal injection of human α-defensins 1-3 mixtures combined with B-cell lymphoma idiotype antigen demonstrated an increased level of antigen-specific IgG antibodies.

In addition, host defence peptides are able to induce apoptosis of macrophages and they activated lymphocytes (Andreu and Rivas, 1998),(Hancock and Diamond, 2000),(Scott and Hancock, 2000). Apoptosis has a major role in the elimination of infected host cells and therefore it decreases the potential replication or survival of viruses or intracellular pathogens. BMAP (bovine myeloid antimicrobial peptide) and lactoferrin demonstrated the capability to enhance apoptosis in macrophage cells lines, transformed cells lines, fresh haematopoietic tumour cells and in vitro-activated human lymphocytes.

1.4 Mode of Actions

It is worth examining the mode of actions of these peptides on bacterial membrane in order to understand how the peptides inhibit microorganisms. The activity and selectivity of host defence peptides are based on the differences between the high concentrations of negatively charged negative charged lipids on the surface of bacterial and eukaryotic cells (Hancock and Diamond, 2000),(Zasloff, 2002),(Kamysz *et al.*, 2003),(Hancock, 2004),(Reddy *et al.*, 2004),(McPhee and Hancock, 2005). Bacterial membranes contain 30% of anionic lipids (phosphatidylglycerol and cardiolipin) and 70% of neutral phospholipids (phosphatidylcholine) on the membrane surface, whereas eukaryotic membranes contain a high proportion of neutral phospholipids (phosphatidylcholine and sphingomyelin) and cholesterol. Furthermore, the anionic components of eukaryotic cells are located along the cytoplasmic side of the membrane. Therefore, the cationic peptides are able to interact with the bacterial membrane, due to a large transmembrane electrical potential gradient generated by the exposed anionic lipid components. This high selectivity on bacterial membrane eliminates the possibility of resistance developments against host defence peptides.

Interaction of host defence peptides with the bacterial membrane causing microbial cell death is not fully understood. Current studies investigating the interaction between peptides and the bacterial membrane have focused more on Gram-negative bacteria with a dual-membrane complex (outer and cytoplasmic). The Gram-negative outer membrane consists of lipids with negatively charged phospholipid headgroups. A model

has been developed, which explains how the peptides interact with bacterial membrane (Hancock, 1997),(Andreu and Rivas, 1998),(Levy, 2000),(Zasloff, 2002),(Powers and Hancock, 2003),(Marshall and Arenas, 2003),(Papo and Shai, 2003),(Reddy *et al.*, 2004),(Brogden, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005). This model describes electrostatic interactions between the peptides and bacterial membrane as the initial interaction. Since the peptides has a high affinity for LPS in the outer leaflet of Gram-negative outer membrane, these cationic peptides are bound to LPS and replace divalent cations, (Ca^{2+} and Mg^{2+}), which are usually present on the outer membrane. The peptides then cause membrane distortion by forming pores in the outer membrane, thus allowing access to the cytoplasmic membrane. At this stage, the peptides can differ into two types of mechanisms: membrane disruptive mechanisms and non-membrane disruptive mechanisms, depending upon the peptides orientation that may lead to perturbation of the bilayer membrane integrity or translocation of the peptides into bacterial cytoplasm (Kamysz *et al.*, 2003),(Powers and Hancock, 2003),(Papo and Shai, 2003),(Brogden, 2005),(McPhee and Hancock, 2005),(Toke, 2005). Host defence peptides are considered membrane active, when they cause membrane disruption at the minimum inhibitory concentration (MIC). The ability of peptides to translocate across the cytoplasmic membrane depends on their secondary and tertiary structures, as well as their oligomeric state.

1.4.1 Membrane Disruptive Mechanisms

Membrane disruptive mechanism takes place when the peptides cause disintegration of the membranes and lead to cell death. There have been three models developed to describe this mechanism, known as barrel-stave, carpet (detergent-like) and toroidal (wormhole) (Hancock, 2001),(Powers and Hancock, 2003),(Papo and Shai, 2003),(Brogden, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005). These three models are shown in Figure 1.7

The barrel-stave model described the formation of barrel-stave like pores, in a way that the hydrophobic sidechains interacted with the lipid core of the membrane, whilst the polar groups pointed inwards producing an aqueous pore (Hancock, 2001),(Powers and Hancock, 2003),(Papo and Shai, 2003),(Reddy *et al.*, 2004),(Brogden, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005). This formation started to take place when the

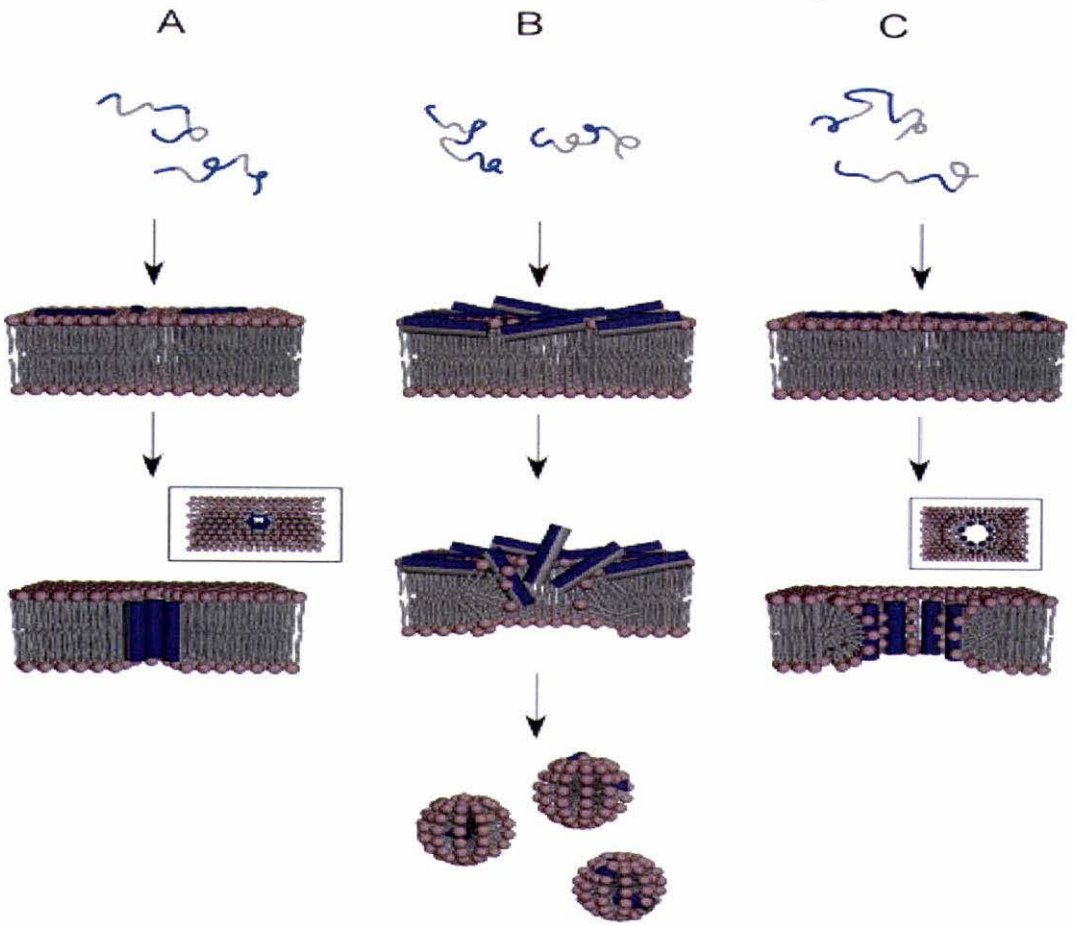
cationic peptide monomers bound in parallel to the cytoplasmic membrane, followed by insertion into the hydrophobic core of the membrane, which was believed to be induced by electrostatic interaction between the anionic surface of the cytoplasmic membrane and the peptides. These monomers tended to oligomerise forming barrel-stave like pores, accompanied by the realignment of the peptides from being parallel to the cytoplasmic membrane to being perpendicular to it (Figure 1.7). Additional monomers were progressively taken up to increases the pore size. The pores caused changes in transmembrane potential and leakage of cytoplasmic components that lead to cell death. However, this model lacks preferred stoichiometries for the pores, due to the wide variability in conductance increases, which were induced by the peptides in the model membranes.

The carpet (detergent-like) model suggested that the peptides, which bound to the outer layer of the cytoplasmic membrane, aligned themselves parallel to the membrane. They formed a coat surrounding a particular area of the membrane, where the peptides were concentrated, in a carpet-like way (Figure 1.7) (Hancock, 2001),(Powers and Hancock, 2003),(Papo and Shai, 2003),(Reddy *et al.*, 2004),(Brogden, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005). Orientation of the peptides was formed in a way that the hydrophobic sidechains faced the inner cytoplasmic membrane, which comprised primarily of phospholipid groups, whilst the polar groups faced out towards the other peptides polar groups. This arrangement would then lead to a disintegration of the area by the disruption of the bilayer curvature, followed by leakage of cytoplasmic components and disruption of membrane potential and eventually the disintegration of the membrane. Some authors refer to this model as the Shai-Matsuzaki-Huang model (Mor, 2000),(Zasloff, 2004),(Sahl *et al.*, 2005). This model, however, was found to be not so accurate, because when host defence peptides were applied at high concentration, many did not depolarise the intact cells leading to cell killing, but instead they tended to translocate across the cytoplasmic membrane and reacted with the cytoplasmic components.

The toroidal (wormhole) model indicated that the host defence peptides were inserted into the cytoplasmic membrane and they induced the phospholipid layers to bend continuously from one membrane leaflet to the other forming a doughnut-like pore (Papo and Shai, 2003),(Brogden, 2005),(Sahl *et al.*, 2005),(Toke, 2005). The wall of this core would be lined with the peptides and the lipid headgroups, because the

membrane surface that already submerged at the hydrophilic/hydrophobic interface got pulled together with the lipid molecules. This model differs from the barrel-stave model, because the peptides are always associated with the lipid head groups even when they are perpendicularly inserted in the lipid bilayer.

Figure 1.7 The three models of host defence peptides membrane disruptive mechanisms on Gram-negative membrane taken from (Toke, 2005). (A) is the barrel-stave model; (B) is the carpet (detergent-like) model; and (C) is the toroidal (wormhole) model.

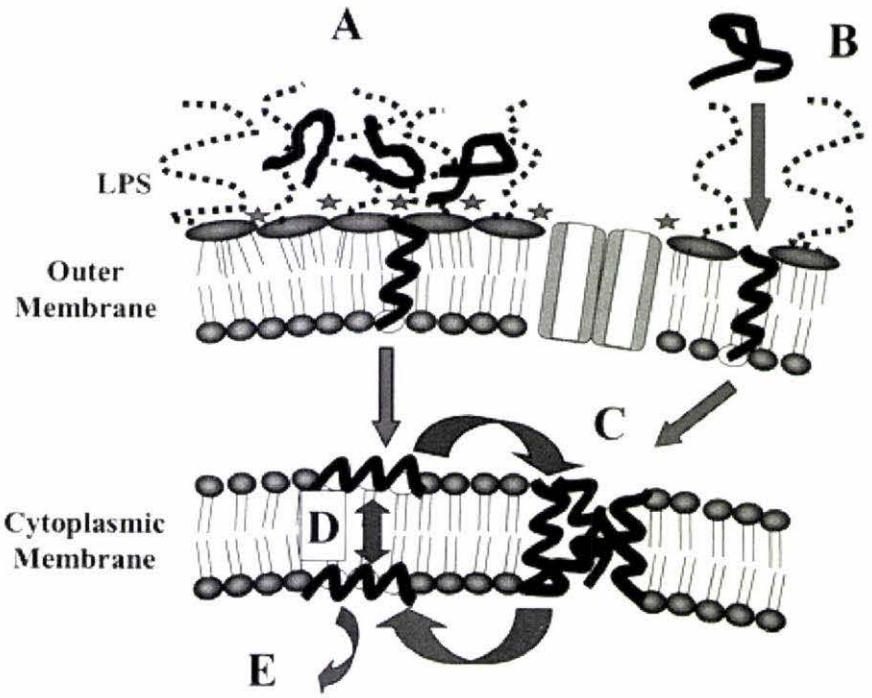


The three models show linear cationic peptides are initially bound to the bacterial membrane. The peptides align themselves along the cytoplasmic membrane, coating the surrounding area. The peptides are subsequently taken up through self-promoted uptake, which then cause the formation of pores that depend on the three models. The pore size can be increased through additional recruitment of peptide monomers.

Further modification to the three models was introduced by Dr Hancock's research group (Hancock and Chapple, 1999),(Hancock, 2001),(Powers and Hancock, 2003). This model is known as a micellar-aggregate mechanism, which was developed to illustrate the capability of host defence peptides to form micellar-aggregates within the

membrane Figure 1.8. This model suggests that the peptides are associated with the cytoplasmic membrane and they insert into a position parallel to the hydrophilic and hydrophobic portions of the membrane. At a critical concentration, the peptides form transmembrane membrane-spanning micellar/aggregate-like arrangements containing lipid and peptide molecules mixtures and they create informal conductance pathways which disturb membrane stability that lead to leakage of protons, ions and larger cytoplasmic. It is also suggested that translocation of the peptides into the cytoplasm takes place if the micellar-aggregates collapse. Some authors include this model as a modification of the carpet model (Sahl *et al.*, 2005).

Figure 1.8 Micellar-aggregate on Gram-negative bacteria (Hancock and Chapple, 1999) (Hancock, 2001),(Powers and Hancock, 2003).

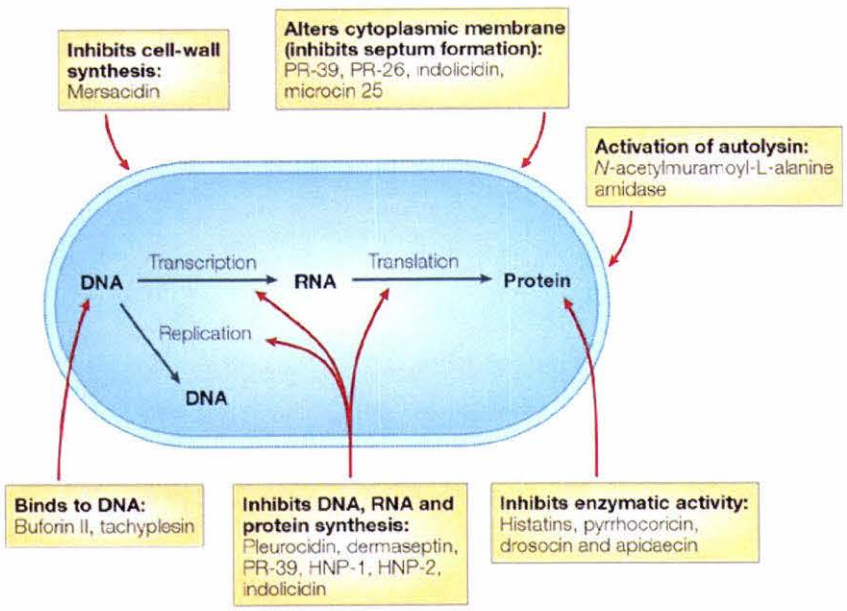


In chronological order, (A) affinity of host defence peptides to LPS displacing the magnesium ions (shown as stars), followed by (B) the partial disruption of the outer membrane allowing the peptides to get into the periplasm. (C) The peptides then cause partial disruption of the cytoplasmic membrane or (D) 'flip-flop' across the membrane to get into the cytoplasm. In the cytoplasm, the peptides are able to interact with intracellular components to inhibit the biochemical reaction of the bacteria.

1.4.2 Non-Membrane Disruptive Mechanisms

There is increasing in vitro evidence that microbial membrane is not the only target of host defence peptides, but these peptides have also been shown to target microbial intracellular components (Andreu and Rivas, 1998),(Epand and Vogel, 1999),(Marshall and Arenas, 2003),(Powers and Hancock, 2003),(Hancock, 2004),(Reddy *et al.*, 2004),(Brogden, 2005),(McPhee and Hancock, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005). Non-membrane disruptive mechanism refers to the interaction between host defence peptides and intracellular targets after the host defence peptides translocate across the membranes into the microbial cytoplasm. This mechanism takes place when disruption is temporary and membrane permeabilisation does not take place (Figure 1.9).

Figure 1.9 Intracellular Targets of Host Defence Peptides in *E. coli* adapted from (Brogden, 2005).



Once in the cytoplasm, these peptides were able to interact with cytoplasmic components, such as DNA, RNA and cellular proteins, which might lead to the inhibition of RNA and protein synthesis, interference with bacterial DNA and the inhibition of DNA replications. Furthermore, they could inhibit the enzyme of a biological pathway in bacteria by forming pseudo-substrates or by binding to the enzyme actives site, thus disturbing access of the real substrates. They could also inhibit

cell wall synthesis and alter cytoplasmic membrane septum formation. In addition, peptides, such as nisin and Pep5, were reported to trigger autolysis, which is a self-destructive mechanism based on activation of amidases that degrade the peptidoglycan in microorganisms (Andreu and Rivas, 1998),(Brogden, 2005),(Otvos Jr., 2005),(Sahl *et al.*, 2005),(Toke, 2005).

Overall, non-membrane disruptive mechanisms can cause both inhibition of cell growth (bacteriostatic) and cell death (bactericidal), whilst membrane disruptive mechanisms of host defence peptides will lead to cell death (bactericidal). However, the cell death caused by non-membrane disruption is much slower (hours after exposure to host defence peptides) than membrane disruptive mechanisms (within minutes of exposure). Since these two mechanisms are present, then it suggests that host defence peptides act synergistically inside the host in killing the pathogens. In addition, it raises a possibility of synergistic usage between the host defence peptides and conventional antibiotics (Scott and Hancock, 2000),(Hancock, 2004),(Otvos Jr., 2005). The synergistic effect, which is based on the interaction between host defence peptides and bacterial membrane, occurs when two different antibiotic compounds act together, resulting in positive cooperation. Furthermore, these multifunctional modes of action indicate that bacteria would have challenging tasks to evolve high-level resistance to these peptides, because there will always be other targets with a higher peptide concentration (Hancock, 2004).

1.5 Defensins

Defensins is a family of host defence peptides that are rich in cysteine residues with amphiphilic β–sheet structures (class I). They are called defensins due to their association with host defence settings. They have been widely distributed in plants (Garcia-Olmedo *et al.*, 1998), insects (Dimarcq *et al.*, 1999), avians, mammalians and humans (Lehrer *et al.*, 1991),(Evans and Harmon, 1995),(Martin *et al.*, 1995),(White *et al.*, 1995),(Brogden *et al.*, 2003),(Lehrer, 2004),(Torres and Kuchel, 2004),(Ganz, 2005). The defensins structure is unique and consists of triple-stranded antiparallel β-sheets with a loop of β-hairpin turn and it is stabilised with three pairs of disulphide bridges formed by six cysteine residues. The cysteine residues are highly conserved in

the defensins family. The defensins positive charge comes from arginine as the predominant cationic residue.

In insects and plants defensins, the structures vary slightly with the addition of an α-helical structure, which is linked to a β-sheet by two disulfide bridges (Garcia-Olmedo *et al.*, 1998),(Dimarcq *et al.*, 1999),(Torres and Kuchel, 2004). Moreover, plant defensins contain four intramolecular disulfide bridges provided by eight cysteines (Evans and Harmon, 1995),(Martin *et al.*, 1995),(Garcia-Olmedo *et al.*, 1998),(Yang *et al.*, 2001). Besides defensins with antimicrobial properties, there are also other peptides with defensins-like-folding with different biological functions. These peptides were found in the venom of platypus, sea anemone and rattlesnake (Zhao *et al.*, 2001),(Ganz, 2003),(Torres and Kuchel, 2004). They are believed to be adaptations of epithelial host defence peptides for efficacy against larger peptides.

1.5.1 Animal and Human Defensins

Animal and human defensins can be divided into three subfamilies: α-, β- and θ-defensins. Amongst these three, α-defensins were firstly isolated from rabbit granulocytes (Selsted *et al.*, 1984), guinea pig granulocytes (Selsted and Harwig, 1987) as well as from human neutrophils (Ganz *et al.*, 1985). The discovery of β-defensins did not happen until the early 1990s, when Michael Selsted's research group successfully isolated a number of β-defensins from bovine neutrophils (Selsted *et al.*, 1993). Lastly, the θ-defensins were isolated from rhesus macaque monkey leukocytes (Tang *et al.*, 1999). Currently, there are more than 50 defensins identified in animal and human (Table 1.2). These peptides are abundant in cells and tissue involved in host defence against microbial infection (Evans and Harmon, 1995),(Martin *et al.*, 1995),(Oppenheim *et al.*, 2003),(Levy, 2004),(Ganz, 2005). The highest concentration of defensins was found in neutrophils and crypts of small intestines (>10mg/ml), whilst in other epithelial cells defensins concentration were in range of 10-100μg/ml. Neutrophils defensins are constantly produced from myeloid cells.

The α-defensins and β-defensins have similar tertiary structures characterised by triple-stranded antiparallel β-sheet (Evans and Harmon, 1995),(Martin *et al.*, 1995),(White *et al.*, 1995),(Yang *et al.*, 2001),(Lehrer and Ganz, 2002),(Boman,

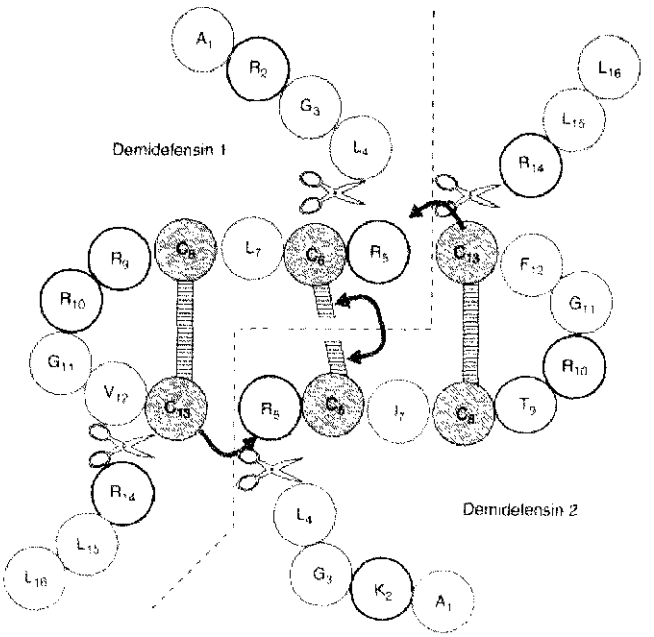
2003),(Ganz, 2005). However, between the first two defensins types, there are distinct variations in the length of peptides and the cysteines spacing and connectivity (Table 1.2). In terms of the length of peptide segments, β-defensins is longer compared with α-defensins, since β-defensins are composed of approximately 38-42 residues, whilst α-defensins consist of approximately 29-35 residues. Furthermore, in α-defensins, the cysteines are paired as 1-6, 2-4, 3-5, whereas in β-defensins, the pattern is 1-5, 2-4, 3-6.

Table 1.2 Comparison of Vertebrate Defensins.

Types	Structure	Size	Residues	Cys Pairings	Source
α-defensins	β-sheet dimer	3.5-4 kDa	29-35	1-6, 2-4, 3-5	human, rabbit, rat, guinea pig, mouse
β-defensins	β-sheet dimer	4-6 kDa	38-42	1-5, 2-4, 3-6	human, bovine, avian turkey, ostrich, chicken, ovine, pig and king penguin
θ-defensins	Cyclic	2 kDa	18	1-4, 2-5, 3-6	rhesus monkey

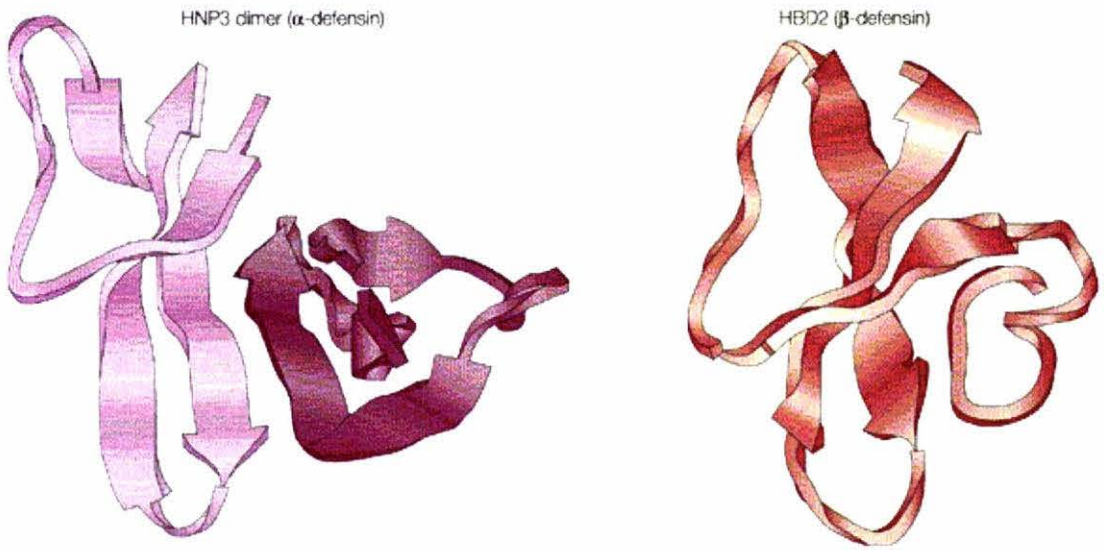
The θ-defensin, which has only been isolated from rhesus macaque monkey leukocytes, is known as the rhesus theta defensin-1 (RTD-1) (Tang *et al.*, 1999),(Lehrer and Ganz, 2002a),(Boman, 2003),(Brogden *et al.*, 2003),(Lehrer, 2004),(Ganz, 2005). This peptide has a distinctive cyclic structure with only 18 residues (Figure 1.10). Formation of RTD-1 was believed to have occurred from the splicing and the cyclisation of two nine-amino-acid segments of α-defensin-like precursor peptides. These two amino acids were translated gene products from two mRNA precursors, which were the products of mutated α-defensin genes with a premature stop codon, resulting in each precursor containing only three cysteine residues (Lehrer and Ganz, 2002),(Ganz, 2003),(Nguyen *et al.*, 2003). In humans, a homologous pseudo gene to θ-defensin, known as retrocyclin-1, was identified but this gene contained a premature stop codon, which prevented it being expressed.

Figure 1.10 Schematic processing of two nine-amino-acid segments of mutated α-defensin precursor into θ-defensins adapted from (Lehrer and Ganz, 2002).



Moreover, humans have six α-defensins and four β-defensins that have been extensively studied (Ryley, 2001),(Gallo *et al.*, 2002),(Donovan *et al.*, 2003),(Oppenheim *et al.*, 2003),(Levy, 2004),(Izadpanah and Gallo, 2005). Four of the human α-defensins (HNP-1-4) were isolated from primary granules (azurophil) of neutrophils and they accounted for 5-7% of the total cellular protein and 30-50% of the azurophil granule protein, whilst two others, HNP-5 and 6, were isolated from intestinal Paneth cells of small intestinal crypts and epithelial cells of the female urogenital tract. The human β-defensins were isolated from epithelial cells of the kidney, urogenital tract, intestines, lung and skin. However, many additional human β-defensins genes, a total of 28, have been identified (Gallo *et al.*, 2002),(Donovan and Topley, 2003),(Brogden *et al.*, 2003),(Lehrer, 2004). Studies by White *et al* (1995) examining structure of human α-defensins and human β-defensins revealed that HNP-1 existed as dimers whilst human β-defensins and other animal α-defensins existed as monomer in solutions (Figure 1.11). The dimers, reportedly shaped as a basket, were formed from monomers that had come into close contact along the edges of their β-hairpins to form a local two-fold rotation axis (White *et al.*, 1995). In this basket, the hydrophobic domain was situated at the bottom and the polar cationic domains were situated at the top.

Figure 1.11 Sequence and Structure of Human Neutrophil Peptide-3 (HNP-3) and Human β-Defensins 2 (HBD-2) seen in (Ganz, 2003).



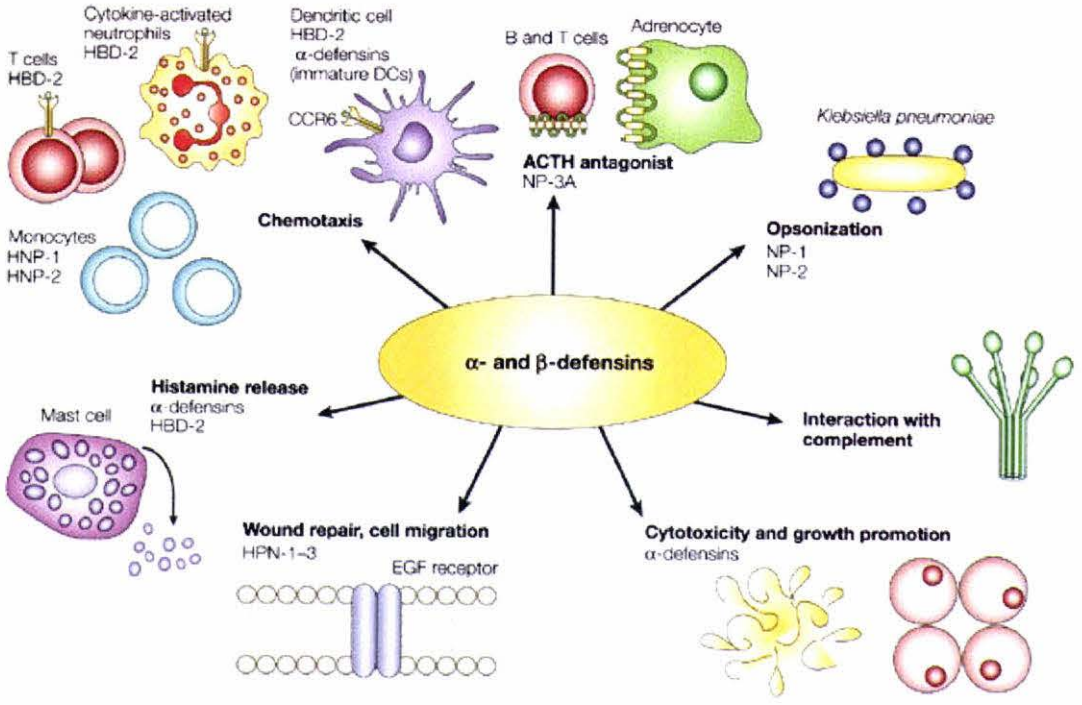
1.5.2 Biological roles

Defensins have a very broad antimicrobial activity spectrum, including Gram-positive and Gram-negative bacteria, protozoans, some fungi, yeasts and enveloped virus, such as human immunodeficiency virus (HIV) and the herpes simplex virus (Lehrer *et al.*, 1991),(Martin *et al.*, 1995),(Yang *et al.*, 2001),(Ganz, 2003). Under optimal conditions, defensins antimicrobial activity was observed at concentrations ranging from 1-10μg/ml. This activity was greatly influenced by the increasing concentration of salts, divalent cations and plasma proteins. Hence, the activity in vivo is likely to occur only in the phagocytic vacuoles of phagocytes, when there are no inhibitors present.

In addition to the direct antimicrobial activity, defensins, in particular human defensins, have demonstrated an ability to interact and enhance innate and adaptive immunity (Figure 1.12) (Yang *et al.*, 2001),(Gallo *et al.*, 2002),(Oppenheim *et al.*, 2003),(Donovan and Topley, 2003),(Lehrer, 2004),(Ganz, 2005),(Izadpanah and Gallo, 2005),(Otvos Jr., 2005). Both defensins can indirectly recruit neutrophils to the inflammation sites, as the defensins enhance chemokine IL-8 and MCP-1 production. This effect results in a positive feedback loop, because the accumulated neutrophils degranulate and release more defensins and these consequently produce more IL-8 and recruit more neutrophils to the inflammation sites. They can also promote mast cell degranulation releasing histamine and prostaglandin D2, indicating a role in allergic

reactions. In addition, human β-defensins are chemoattractant to T-cells and the dendritic cells to the inflammation sites, due to the defensins binding to the chemokine receptor (CCR6), whereas human α-defensins demonstrate a chemotactic ability to monocytes, T-cells, naïve cells and immature dendritic cells to the inflammation sites.

Figure 1.12 Interaction of Defensins with Immune System adapted from (Lehrer, 2004).



Human β-defensins also promote dendritic cells maturation. The defensins promote migration of immature dendritic cells from inflammation sites to lymph nodes, where maturation of dendritic cells takes place. As the dendritic cells mature, they are able to process antigens and display responses on their surfaces in the form of antigen-MHC II complexes. Furthermore human β-defensins are involved in stimulation of the Toll-like receptor-4 (TLR-4) that amplifies an innate response. On the other hand, human α-defensins are able to enhance phagocytosis by macrophage, which stimulate wound healing and induce an antigen-specific immune response. The expression of TNF-α and IL-1 in monocytes can also be induced by human α-defensins, which indirectly stimulate maturation of dendritic cells, even though they are not chemoattractant to mature dendritic cells. Furthermore, they can enhance or suppress the classical pathway

of the complement cascade system in vitro, indicating a possibility that human α-defensins play a role in regulating the complement cascade system.

1.6 Purification and Characterisation of Avian β-Defensins

Avian host defence peptides are composed of mainly β-defensins, which have been purified and characterised from myeloid (heterophil) and non-myeloid cells (non-heterophil). The heterophil peptides were successfully purified from chicken, turkey and ostrich heterophils (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001). The non-heterophil peptides were obtained from expressions from epithelial β-defensins of chicken and turkey (Zhao *et al.*, 2001) and isolation from king penguin stomach content (Thouzeau *et al.*, 2003). There were also chicken β-defensins identified from chicken genome (Lynn *et al.*, 2004),(Xiao *et al.*, 2004). Table 1.3 shows the alignment of the amino acid sequences of avian heterophil and non-heterophil peptides with two bovine β-defensins isolated from neutrophils and epithelial cells, and a β-defensin from human epithelial cells. These β-defensins sequences are highly diverse but they share eight conserved residues, the six-cysteines and two glycines (glycine^{11} and glycine^{26}).

Avian β-defensins are classified in the third class amongst the β-defensins peptides (Zhang *et al.*, 1998). This classification, which is based on the length and homology of peptide and gene structures, is divided into the first class, containing a short prepro-sequences (63-64 residues) and short introns (less than 1.6 kbp), the second class, containing a long prepro-sequences (68-69 residues) with more than 6.5 kbp of introns, and the third class, which is particularly for avian β-defensins.

Table 1.3 Amino acid sequences of existing avian and mammalian β-defensins.

Source	Peptide	Amino Acid Sequence				
		1	10	20	30	40
Chicken	CHP-1[□]	GRKSDCFRKS	GFCAFLKCPS	LTLISGKCSR	FYL-CCKRIR	
	CHP-2[□]	GRKSDCFRKN	GFCAFLKCPY	LTLISGLCSX	FHL-CC	
	Gal-1[□]	GRKSDCFRKS	GFCAFLKCPS	LTLISGKCSR	FYL-CCKRIW	
	Gal-1α[□]	GRKSDCFRKN	GFCAFLKCPY	LTLISGKCSR	FHL-CCKRIW	
	Gal-2[□]	---LFC--KG	GSCHFGGCPS	HLIKVGSCFG	FRS-CCKWPW	NA
Ostrich	OSP-1[□]	---LFC--RK	GTCHFGGCPA	HLVKVGSCFG	FRA-CCKWPW	DV
Turkey	THP-1[□]	GKREKCLRRN	GFCAFLKCPT	LSVISGTCSR	FQV-CC	
	THP-2[□]	---LFC--KR	GTCHFGRCPS	HLIKVGSCFG	FRS-CCKWPW	DA
	THP-3[□]	---LSC--KR	GTCHFGRCPS	HLIK-GSCSG	G	
Chicken	Gal-3[◊]	GTATQCRIRG	GFCRVGSCRF	PHIAIGKCA-	TFISCCGRAY	
Turkey	GPV-1[◊]	GTPIQCRIRG	GFCRFGSCRF	PHIAIAKCA-	TFIPCCGSIW	
King Penguin	Sphe-1[Δ]	-SFGLCRLRR	GFCAHGRCRF	PSIPIGRCS-	RFVQCCRRVW	
	Sphe-2[Δ]	-SFGLCRLRR	GFCARGRCRF	PSIPIGRCS-	RFVQCCRRVW	
Bovine	TAP[▲]	-NPVSCVRNK	GICVPIRCPG	SMKQIGTCVG	RAVKCCRKK	
	BNBD-1[•]	-DFASCHTNG	GICLPNRCPG	HMIQIGICFR	PRVKCCRSW	
Human	HBD-1[▲]	-DHYNCVSSG	GQCLYSACPI	FTKIQGTCYR	GKAKCCK	
Consensus		-----C-----G-C----C--------G-C-------CC-------				

These β-defensins were isolated from heterophils (□), epithelial (◊), stomach content (Δ) of avian species as well as human epithelial peptide (▲), bovine tracheal peptide (▲) and bovine neutrophil peptide (•). The consensus is found in all the sequences except in GPV-1, where glycine[26] is replaced with alanine[26] (blue highlighted).

1.6.1 Avian Heterophil β-Defensins

Avian heterophil peptides include: two chicken heterophil peptides (CHP-1 and CHP-2 (Evans *et al.*, 1994)); three gallinacins from chicken (Gal-1, Gal-1α and Gal-2 (Harwig *et al.*, 1994)); three turkey heterophil peptide (THP-1, THP-2 and THP-3 (Evans *et al.*,

1994)); and ostricacin from ostrich (Osp-1 (Yu *et al.*, 2001)). These β-defensins are rich in arginine, lysine and cysteines. The molecular weight of Gal-1, -1α, -2, and Osp-1, as determined with electrospray ionisation mass spectrometry (ESI-MS), are 4582, 4505, 3915.58 and 4011.55 Da respectively (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001). The molecular weights of the turkey peptides were not determined.

Based on amino acid sequences, the avian heterophils β-defensins can be separated into two sub-classes. The first sub-class has a longer sequence with 35-39 amino acids and shares a total of 22 identical amino acids, whilst the second sub-class, with the exception of THP-3, is comprised of 36 amino acids and shares 17 identical residues. Peptides in the first sub-class are THP-1, CHP-1, CHP-2, Gal-1 and Gal-1α, whilst peptides in the second sub-class are THP-2, THP-3, Gal-2 and Osp-1. Between the two sub-classes, they share nine identical residues, which are the eight conserved residues with the addition of a proline[19]. In addition, the second sub-class is characterised by three missing amino acids at the N-terminus followed by the leu-phe/leu-ser sequence. On the other hand, the first sub-class begin with glycine followed with two basic residues (arginine or lysine). Another noticeable variation is the second sub-class, which contains only four residues between its first and second cysteine, instead of six residues, as in the first sub-class.

Analysis of the peptides in the first sub-classes showed that there are inter-molecular similarities that can be observed between two prepro-peptides of gallinacins (Gal-1 and 2) and two THPs (THP-1 and 2). These similarities were demonstrated in a study examining the amplification of chicken and turkey antimicrobial peptides cDNA from their respective bone marrow (Brockus *et al.*, 1998). The cDNA of these β-defensins was comprised of 409-494 nucleic acid residues that translated into peptides with 64-65 amino acid residues. The prepro-peptides of Gal-1 and THP-1 contained 65 amino acids with 85% similarity, whereas Gal-2 and THP-2 prepro-peptides contained 64 amino acids with 93.2% similarity. Furthermore, the β-defensins cDNA sequence composition demonstrated more similarity to the bovine TAP and LAP, than the other mammalian classical defensins.

In addition, the study concluded that there were similarities between Gal-1 and CHP-1, as well as between Gal-1α and CHP-2, which could be due to genetic heterogeneity or actual cleavage of the terminal amino acid from the mature peptide. The mature peptide

sequences, generated from the cDNA amplification of CHP-1 and Gal-1, ended with glycine at the C-terminus, instead of it being arginine or tryptophan residues as shown in Table 1.3. Apart from this variation at the C-terminus, both sequences were almost homologous. The other two peptides, Gal-1α and CHP-2, also showed striking similarities with differentiations were in the lengths and the two residues. Gal-1α showed four more residues on the C-terminus than CHP-2 and the two residues variation were Lys^{27}/Leu^{27} and Arg^{30}/undetermined. Therefore, Brockus *et al.* (1998) concluded that these chicken peptides were identical and called themGal1/CHP1 and Gal1α/CHP2, respectively. Gal1/CHP1 and Gal1α/CHP2 were almost identical with variation in only three positions, Ser^{10}/Asn^{10}, Ser^{20}/Tyr^{20} and Tyr^{32}/His^{32} (Harwig *et al.*, 1994).

1.6.2 Avian Non-Heterophil β-Defensins

The two epithelial β-defensins, known as gallinacin-3 (Gal-3) and gallopavin-1 (GPV-1), were expressed from the tracheal of chicken and turkey, respectively (Zhao *et al.*, 2001). Both peptides were comprised of 39 amino acid residues. The expression of Gal-3 in trachea was significantly affected by *Haemophilus paragallinarum*. Comparison of cDNA, amplified from the bone marrow, between the two peptides showed 91% similarity. Meanwhile, compared with the avian heterophils peptides, these epithelial peptides shared more identical residues with the first sub-class. The mature epithelial peptide of Gal-3 showed the same number of residues as Gal1/CHP1 and Gal1α/CHP2 but only thirteen identical residues were shared between them, whereas mature GPV-1 showed longer amino acids than THP-1 and shared twelve identical residues. Gal-3 cDNA, in contrast, showed approximately 75% overall identity to the Gal1/CHP1 and Gal1α/CHP2 cDNA, which was most marked in their signal sequence and 3'untranslated regions. Likewise, the comparison of GPV-1 and THP-1 cDNA appeared 83% identical mostly at the signal sequence and 3'untranslated regions. Zhao *et al* (2001) also found that Gal1/CHP1, Gal1α/CHP2 and Gal-2 were expressed strongly in healthy bone marrow and in lungs, whereas Gal-3 was expressed in non-myeloid cells, including a strong expression in tongue, Bursa of Fabricius, trachea, moderate expression in skin, oesophagus and air sacs and weak expression in the large intestine, kidney, ovary and bone marrow.

Recently, thirteen β-defensins gene encoding for gallinacins-1-13 were identified in the chicken genome, when defensins sequences from plant, invertebrates and vertebrates were used to search GenBank using a TBLASTN program (Xiao *et al.*, 2004). Three of these β-defensins (Gal-1-3) had been previously known (Harwig *et al.*, 1994),(Zhao *et al.*, 2001), whilst nine gallinacins (Gal-4-12) were newly discovered in the expressed sequence tag (EST) database. The gallinacins-13 was retrieved when high throughput genomic sequence (HTGS) and whole genomic sequence (WGS) containing defensins sequences were translated into six open reading fames and manually curated. All thirteen sequences were comprised of 63-104 amino residues containing hydrophobic signal sequence that was rich in leucine at the N-terminus, a short propiece sequence and a cationic mature peptide characterised with the six cysteine defensin motif and rich in arginines and lysines. The signal peptides sequences were highly conserved with slight variations, whilst the mature peptides sequences were highly diverged. In particular, Gal-11 contained two tandem copies of the six-cysteine motif at the C-terminus. Tissue expression of these gallinacins revealed that Gal-1-7 were mainly expressed in bone marrow and the respiratory tract and the remaining gallinacins (Gal-8-13) were mainly expressed in the liver and the urogenital tract. A weak expression of Gal-5 was also observed in the tongue, trachea, lungs and brain.

A similar approach, using BLAST search and a hidden Markov model (HMM) resulted in the identification of seven β-defensins, named gallinacins-4-10 (Lynn *et al.*, 2004). Even though the sequences were the same as reported in Xiao *et al.* (2004), the gallinacins naming was not the same. Lynn *et al.*(2004) also identified a gene encoding for a cathelicidin, which has not been discovered in any other avian species, and a liver-expressed antimicrobial peptide-2 (LEAP-2), which characteristic was not described.

In addition to the chicken and turkey β-defensins, spheniscins-1 and 2 (Sphe-1 and 2) are the most recent β-defensins isolated from stomach contents of a different bird species, king penguins (Thouzeau *et al.*, 2003),(Landon *et al.*, 2004). The successful isolation was followed with an investigation into the solution structure of Sphe-2 (Landon *et al.*, 2004). These peptides were found at higher concentrations during the conserving period than that in digesting birds, indicating the importance of conserving the male penguin's stomach contents, which is used for feeding the newly-hatched chicks, during the time when the female bird has not returned from foraging at sea.

Both, Sphe-1 and Sphe-2, are comprised of 38 amino acid residues with molecular weights of 4482.4 and 4501.4 Da, respectively. The small differences in molecular weight were associated with the differences of histidine/arginine residue at position 14. This differentiation on the isoforms was thought to be due to gene polymorphism. Sphe-2 structure, which was successfully determined using two dimensional NMR and molecular modelling techniques, appeared to be a triple stranded antiparallel β-sheet, stabilised by three disulfide bridges with a typical β-defensins pairing in solution.

Compared to other avian β-defensins, sphenisins are more positively charged, with a net charge of +10 (Thouzeau *et al.*, 2003),(Landon *et al.*, 2004). The positive charge of Gal1/CHP1, Gal-2, THP-1 and THP-2 are +8, +6, +7, and +7, respectively (Brockus *et al.*, 1998). Moreover, the structure of Sphe-2 showed a hydrophobicity patch that was not preserved in other avian and mammalian β-defensins. When sphenisins sequences were compared with other avian β-defensins, they shared more similarities to the avian epithelial β-defensins, than the avian heterophil β-defensins. The sphenisins retained 50% similarity to the chicken Gal-3 and 47% similarity to the turkey GPV-1, whilst the percentage similarity to Gal1/CHP1, Gal1α/CHP2, THP-1 and THP-2 was only 37-39%. Comparison of sphenisins to Gal-2 showed the least similarity at 33%. On the contrary, sphenisins shared several residues with the avian β-defensins, including a basic residue (Arg^{9} or Lys^{9}), a hydrophobic residue (Ile^{23}, Leu^{23} and Val^{23}), $glycine^{26}$, $phenylalanine^{32}$, two hydrophobic residues (Val^{39} and Trp^{40}) at the C terminus and the seven conserved residues.

1.6.3 Antimicrobial Activity of Avian β-Defensins

Most β-defensins have shown antimicrobial activity against a wide range of pathogens including bacteria and fungi. Avian β-defensins play a more important role in the innate defence system, because of the avian heterophil lack of oxidative mechanisms (Topp and Carlson, 1972),(Montali, 1988). An oxidative mechanism consists of superoxide ion, hydrogen peroxide and myeloperoxidase, whilst a non-oxidative mechanism consists of a few enzymes, cationic proteins and peptides. Even though they lack oxidative mechanisms, studies examining chicken and turkey heterophils have shown microbicidal activity, indicating that lysozymes and the cationic proteins/peptides would be the main mechanisms (Brune *et al.*, 1972),(Brune and Spitznagel,

1973),(Harmon *et al.*, 1992),(Evans *et al.*, 1994),(Harmon, 1998). Since these cationic peptides may be the primary mechanism for avian heterophil antimicrobial activity, a variety of peptides present in heterophils, together with the bactericidal activity of lysozymes and other proteins, would define the activity spectrum of heterophils.

Previous research work undertaken by Brune *et al* showed the ability of chicken heterophils to inhibit growth of *Escherichia coli, Staphylococcus albus, Candida albicans* and *Serratia marcescens* (Brune *et al.*, 1972),(Brune and Spitznagel, 1973). At least three cationic proteins or peptides from the heterophil granule extracts were reported, but none of them were isolated and characterised. Recent research works into avian β-defensins showed antimicrobial activity against a number of microorganisms (Table 1.4). For comparative purposes, the activity bovine neutrophil β-defensins (BNBD-1) and rabbit neutrophil peptide (NP-1) are included.

Table 1.4 Antimicrobial activity of avian β-defensins.

Peptide	Spectrum of Activity
Gal-1 Gal-1α	*Candida albicans, Escherichia coli* and *Listeria monocytogenes*
Gal-2	*Escherichia coli* and *Listeria monocytogenes*
CHP-1 CHP-2	*Candida albicans, Escherichia coli, Bordetella avium, Salmonella enteritidis, Salmonella typhimurium,*
THP-1	*Campylobacter jejuni* and *Mycoplasma gallisepticum*
THP-2	*Staphylococcus aureus*
THP-3	*Candida albicans, Salmonella enteriditis* and *Campylobacter jejuni*
Osp-1	*Escherichia coli* and *Staphylococcus aureus*
Sphe-2	*Bacillus subtilis, Escherichia coli, Staphylococcus aureus* and *Aspergillus fumigatus*
BNDBD-1	*Escherichia coli* and *Staphylococcus aureus*
NP-1	*Escherichia coli* and *Staphylococcus aureus*

The majority of avian heterophil β-defensins have antimicrobial activity against Gram-positive bacteria, e.g. *Staphylococcus aureus*, and Gram-negative bacteria, e.g. *Escherichia coli*. Only some peptides (Gal1/CHP1, Gal1α/CHP2, THP-1 and THP-3) have demonstrated antimicrobial activity against *Candida albicans* (Harwig *et al.*,

1994),(Evans *et al.*, 1995). These antimicrobial activities were determined in conditions of low ionic strength and low concentrations of interfering substances. In contrast to the avian heterophil, synthetic spheniscin-2 showed antimicrobial activity against a wider range of microorganisms (Thouzeau *et al.*, 2003),(Landon *et al.*, 2004). It showed bactericidal against all Gram-positive bacteria tested, except *Staphylococcus saprophyticus*, but mainly showed bacteriostatic against Gram-negative bacteria tested, apart from *Escherichia coli* 1106 and *Vibrio metsnikovii*, for which Sphe-2 was bactericidal. Spheniscin-2 also showed an inhibition of *Aspergillus fumigatus* sporulation, but it did not show activity against *Candida sp.* The synthetic Sphe-2 also appeared to retain antimicrobial activity at low pH and high salt concentration, which was the condition of the king penguin's stomach contents. The differences between Sphe-2 antimicrobial activity and the other avian β-defensins were believed to be due to the structural properties of the peptides. As described earlier, Sphe-2 is more cationic and contains a hydrophobic patch that was not preserved in other β-defensins.

1.6.4 Purification and Characterisation Techniques of Avian Heterophil β-Defensins

Purification of host defence peptides from blood involves a number of steps. The first step is the extraction of the peptides from the heterophil blood and this is followed with a series of purification steps to isolate individual peptides. The isolated peptides are then tested for antimicrobial activity. Once confirmed to be antimicrobial, the sequence and structures of the peptide can be determined.

There have only been three different publications showing a successful isolation of β-defensins from avian heterophils (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001). The publication by Yu *et al.* (2001) was the first publication concerning the isolation of host defence peptides from animal blood in New Zealand. In all the publications, the extraction of avian peptides from blood involved removal of the red blood cells, disruption of the granulocytes and acid extraction of host defence peptides from the granules. Each step was followed by subsequent centrifugation in order to collect the desired materials. The first step was usually the separation of red blood cells from the plasma using centrifugation, which was then followed by the disruption of red blood cells with cold deionised water. Instead of cold deionised water, the use of 0.83%

ammonium chloride for disrupting the red blood cells has been reported (Eggleton *et al.*, 1989), (Anderson and Yu., 2003). The granulocytes were then separated from the lysed erythrocytes with centrifugations and collected in a buffer. A few buffers were used to resuspend the white blood cells, including a 0.05M sodium phosphate buffer containing 2mM EDTA, 10μM leupeptin and 10μM pepstatin (Evans *et al.*, 1994), a PBSX buffer containing 137mM NaCl, 2.7mM KCl, 0.5mM $MgCl_2$, 8.1mM Na_2HPO_4 and 1.5mM KH_2PO_4 (Anderson and Yu., 2003).

The next step was the disruption of white blood cells. The most common methods of disruption of heterophils are homogenisation and sonication, of which sonication is the most favoured methods, as shown in the disruption of chicken and turkey heterophils (Evans *et al.*, 1994), ostrich heterophils (Yu *et al.*, 2001), ovine neutrophils (Anderson and Yu., 2003), rabbit neutrophils (later experiments) (Selsted *et al.*, 1984). Homogenisation was reported in the disruption of chicken heterophils (Harwig *et al.*, 1994), pig neutrophil (Selsted and Harwig, 1987) and rabbit neutrophils (Selsted *et al.*, 1984). In bovine neutrophils, the disruption was carried out using nitrogen cavitation in a Parr bomb (Selsted *et al.*, 1993). Once the granules were separated, the peptides could be extracted using acid extraction. The most common acid used is acetic acid (Selsted and Harwig, 1987),(Selsted *et al.*, 1993),(Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001),(Anderson and Yu., 2003)

For the purification of host defence peptides from crude extract, there have been a few different techniques used, including ion-exchange chromatography, gel filtration and reverse phase high performance liquid chromatography (RP-HPLC) (Selsted, 1997). The first purification step of avian peptides from the crude extract was gel filtration using Acrilex P-10 (Harwig *et al.*, 1994), Biogel P-10 (Evans *et al.*, 1994) or Biogel P-60 (Yu *et al.*, 2001). The use of gel filtration was also reported in the purification of β-defensins from bovine neutrophils (Selsted *et al.*, 1993). The eluant used in all gel filtration was 5% (v/v) acetic acid. The use of cationic exchanges, such as Bio-Rad Macro-Prep® CM, was suggested (Brogden, personal correspondence, 2002). Reverse phase high performance liquid chromatography (RP-HPLC), with linear gradient containing a mixture of water, acetonitrile and 0.1% trifluoroacetic acid (TFA), followed the gel filtration step in order to isolate the individual peptides. The use of RP-HPLC has been reported in virtually every modern study involving peptides isolation and purifications (Shaw, 1994),(Selsted, 1997). This technology has also been

used to purify host defence peptides from animal granulocytes (Selsted and Harwig, 1987),(Selsted *et al.*, 1993),(Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001),(Anderson and Yu., 2003).

A technique used to test the antimicrobial activity of the avian peptides is the radial diffusion plate assay method, which was developed by Dr Lehrer's research group (Lehrer *et al.*, 1991),(Steinberg and Lehrer, 1997). In this assay, the microbes were added to an underlay agar that lacked nutrients. The peptides were added to wells, made in the underlay agar, and left to diffuse. An overlay agar, which was full of nutrients, was added to the top of the underlay agar. The microbes grew between the two layers of agar, but if the peptides were antimicrobial, they inhibited the growth, which was indicated by a clearing of the grown agar. This technique was used to test antimicrobial activity on the crude extract and the fractions of gel chromatography and RP-HPLC as well as to determine the minimum inhibitory concentration (MIC) of the peptides (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001). The MIC is the minimum concentration of peptides required to reduce the bacterial concentration to less than 10% of the original inoculum. To determine the MIC, different concentrations of the peptides are used in the radial diffusion assay and then the diameter of the clearing sizes are plotted against a log of the peptide concentration. A straight line is then fitted and from the equation of the line the x-intercept is calculated as the MIC.

Once the antimicrobial properties of the peptide had been purified, the structures were determined. In the avian β-defensins, from heterophil or other origins, mass and amino acid sequence of the peptides were determined using mass spectrometry and N-terminal sequencing, respectively (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001), (Thouzeau *et al.*, 2003). Only the structure of sphenisein-2 has been determined, the other avian β-defensins structure has not been predicted but, from the amino acid sequence, they are known to retain the triple antiparallel β-sheets structure (Landon *et al.*, 2004).

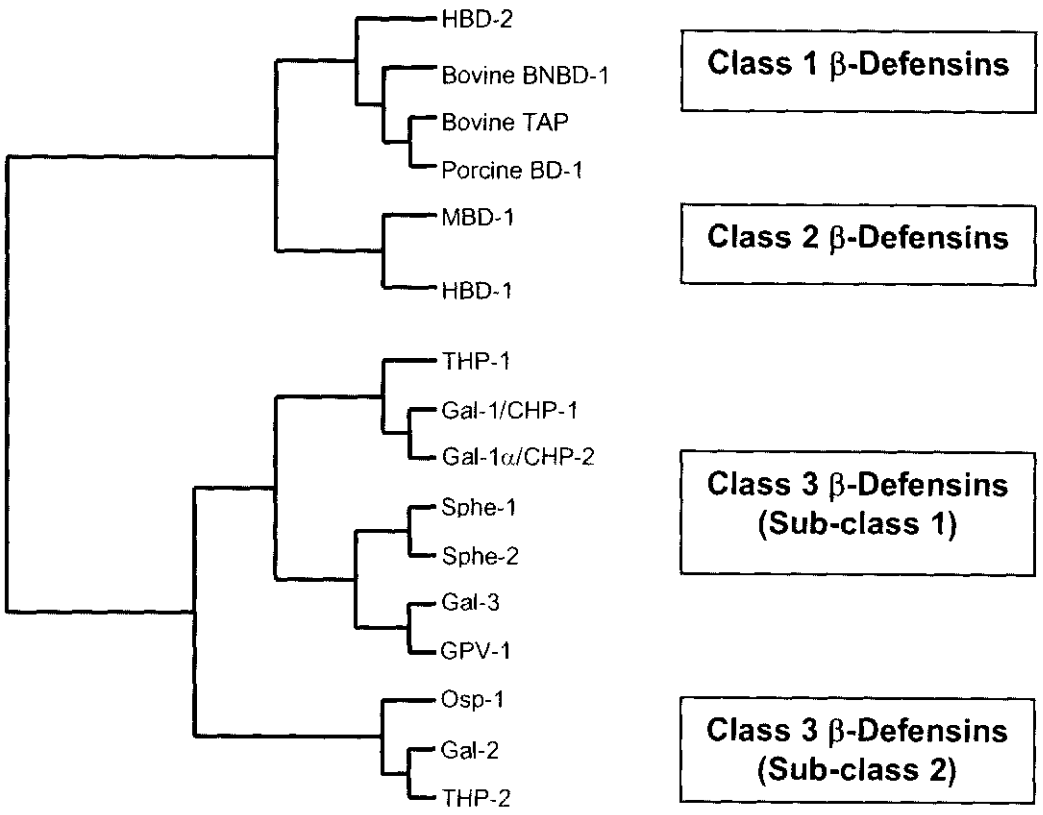
1.7 Evolutionary of β-Defensins

The β-defensins are translated from genes containing two exons: exon-1 encodes the signal peptide and exon-2 encodes the propiece and mature defensin domain. Even

though, β-defensins were found widely in almost all vertebrates, only the signal sequence is highly conserved; the propiece sequences and mature peptides sequences tend to be widely diverse, apart from the cysteines framework. The extensive variations are believed to be due to a positive selection on the short length of β-defensin. Morrison *et al.* (2003) calculated the rate of nonsynonymous substitution (d_N) and the rate of synonymous substitution (d_S) per site, for sequences of paralogous genes, within fourteen murine and four human β-defensin genes showed that following gene duplication, exon-1 and surrounding non-coding DNA had little divergence within subfamilies, whilst exon-2 had substantial variation with a ratio d_N/d_S greater than 1, indicating the positive Darwinian selection (Maxwell *et al.*, 2003). This positive selection was also shown in the avian β-defensins (Brockus *et al.*, 1998),(Zhao *et al.*, 2001),(Lynn *et al.*, 2004).

The broad distribution of β-defensins across vertebrates species, whilst the other two types of defensin were restricted to mammalian, indicated a possibility that the β-defensins were the oldest defensin subfamily (Hughes, 1999),(Zhao *et al.*, 2001),(Lehrer and Ganz, 2002),(Nguyen *et al.*, 2003). A phylogenetic tree diagram (Figure 1.13), generated by using the Clustal X program, showed the clustering of avian β-defensins with selected mammalian β-defensins. The phylogenetic tree agrees with the classification proposed by Zhang *et al.* (1998) with a modification in the avian β-defensins that is divided into two sub-classes. Since the divergence of mammalian and avian was believed to have taken place approximately 310 million years ago (Hedges *et al.*, 1996),(Kumar and Hedged, 1998), the clustering of avian and mammalian β-defensins indicated that the β-defensins gene must have existed before the last common ancestor of birds and mammals (Zhao *et al.*, 2001). This evidence is strongly supported with the purification of Osp-1 from ostrich, a group of ratite families that belongs to Palaeognathiformes, the oldest order of living birds. It has been reported that the divergence within ratites was not earlier than 90 million years ago, due to the break up of super-continent Gondwana around 150 million years ago (Cooper *et al.*, 1992),(van Tuinen *et al.*, 1998). Therefore, β-defensin genes would have been around for at least 90 million years.

Figure 1.13 Evolutionary tree of existing β-defensins.



This evolutionary tree was constructed from alignment of chicken gallinacins (Gal-1-3/CHP-1-2), turkey heterophil peptides (THP-1-2) and epithelial peptide (GPV-1), ostrich peptide (Osp-1), king penguin spheniscins (Sphe-1-2), human β-defensins (HBD-1-2), mouse β-defensin (MBD-1), porcine β-defensin (BD-1) and bovine β-defensins (BNBD-1 and TAP).

The second evidence, showing that β-defensins are the oldest defensin subfamily, was provided when striking similarities between vertebrate β-defensins and several peptide of animal venoms were drawn (Zhao *et al.*, 2001),(Lehrer and Ganz, 2002),(Ganz, 2003),(Nguyen *et al.*, 2003),(Torres *et al.*, 2004). These venoms that retain β-defensins motifs were crotamine from South American rattlesnake, anthopleurins, BDS and Sh1 from sea anemones, and four defensins-like-peptides (DLPs) from male duck-billed platypus venom. The DLPs and the crotamine showed conserved sequences with Gal-3, GPV-1 and human β-defensins-3. The similarities were also shown by platypus DLPs, which were tertiary structures resembling bovine neutrophil β-defensins-1 (BNBD-1). Furthermore, DLPs and Sh1 contained a short N-terminal helix that resembled α-helical segments in human β-defensins-1 and 2. Modern mammals are descended from reptiles (therapsids), which diverged from other reptilians as long ago as 220 million years, at

the same time as avian lines deviated from reptilians. The structural and sequence similarities of these compounds in the reptilians indicated that they genetically originated from an ancient β-defensin-like gene. The difference in function was believed to be due to adaptation of epithelial host defence peptides for efficacy against larger predators.

In addition, comparison between α-defensins, β-defensins and insect defensins, based on the proportion of amino acid differences, showed that β-defensins were more closely related to the insect defensins, than to the mammalian α-defensins (Hughes, 1999). Between α- and β-defensins, five of the six-cysteine residues were the only conserved amino acid residues, whereas β-defensins and insect defensins conserved all six-cysteine residues. Within α- and β-defensins, positive Darwinian selection was displayed, when the rate of d_N exceeded the rate of d_S in the region of the gene encoding the mature defensins. Overall, the three defensins were distantly related as they shared common similarities only in the cysteine residues, suggesting that they evolved a similar convergent structure from gene duplication. Furthermore, this study, demonstrated that α-defensins had repeatedly duplicated after the divergence of these species, as a phylogenetic tree showed that α-defensins formed species-species clusters. Mammalian α-defensins also showed a unique coordinated evolution, such that the charge changes in the propieces tended to be accompanied by an opposite charge change in the mature peptides, which caused little change in the net charge (Hughes and Yeager, 1997),(Hughes, 1999), (Lynn *et al.*, 2004). The rapid evolution of α-defensins was believed to be due to the evolutions of mammals, which occupied new niches and faced new pathogens.

1.8 Applications of Host Defence Peptides

There is a growing need to develop new classes of antimicrobial agents, with increasing resistance in bacterial species to conventional antibiotics (Zasloff, 2002),(Brogden *et al.*, 2003),(Falla and Zhang, 2004),(Kues and Niemann, 2004),(Reddy *et al.*, 2004),(Otvos Jr., 2005),(Zhang and Falla, 2006). Furthermore, it has been more than 30 years since a truly new class of antibiotics, linezolid, was introduced. The highly active nature of host defence peptides appeared to be the answer to the problem, because their

mode of action focuses on the permeabilisation of microbial membranes and interaction with microbial cytoplasmic targets, instead of specific microbial targets, which is the mode of action of most conventional antibiotics. Since the microbial membrane composition is similar between species and strains, the microorganisms are less likely to develop resistance against the non-specific action of host defence peptides. In addition, the wide spectrum of antimicrobial activity and their roles in the immunity of the host defence peptides indicated that they are effective to treat bacterial infection and enhance immune responses. However, these peptides are limited by their low efficacy and their inadequate safety margins, making them suitable only for topical applications.

1.8.1 Current Developments of Host Defence Peptides into Therapeutic Applications

With declining supplies of appropriate anti-infective agents, many large pharmaceutical companies withdrew from the field of antibiotic research and development. Several host defence peptides have been developed in small biotechnology companies. These developments are mostly to commercialise the work of their leading researcher. Some of these peptides are currently undergoing laboratory testing but few have reached clinical trials. In the following section, the small biotechnology companies are outlined together with their leading products and clinical trials results.

The first company, Demegen Inc., is based in Pittsburgh, USA and it has two leading products, Demegel™ and Histawash™ or Histagel™ (Reddy *et al.*, 2004),(Hancock, 2000),(Falla and Zhang, 2004),(McPhee and Hancock, 2005). Demegel™ is a gel formulation used as a wound-healing product for infected burns and wounds that has been developed from a 22-residue-α-helical peptide. This peptide demonstrated a wide spectrum of antimicrobial spectrum including resistant strains of *P. aeruginosa*, *S. aureus* and antifungal activity. The other two products are developed from P-113, a 12-residue portion of histatins, which are compounds found naturally in human saliva. Histagel™ and Histawash™, were developed from an L form of the peptide that has a potent activity against *Candida sp.* and they are used as mouth rinse formulation for the treatment and prevention of gingivitis. The D form of P-113, which showed potent activity against clinical isolates *P. aeruginosa* of cystic fibrosis (CF) patients, have been developed for therapy against lung infections in chronic CF patients. Demegel™ and

Histawash™ have undergone phase II clinical studies whereas the P-113D have just started preclinical studies.

The second company, Genaera Corporation located in Plymouth, USA, is the owner of Magainin Pharmaceuticals' peptide development programs, which was founded to commercialise host defence peptides discovered by Michael Zasloff (Zasloff, 2002),(Falla and Zhang, 2004),(McPhee and Hancock, 2005),(Zhang and Falla, 2006). The leading product of Genaera is pexiganan, also known as Locilex™ or MSI-78. It is a 22-residue variant of the amphibian peptide magainin-2, with a slight modified C-terminus that improves its spectrum activity and increases its stability. Pexiganan is intended for treating mild or moderate infections of diabetic foot ulcers. This product had reached phase III clinical trial before failing, because it could not offer greater benefit than the current standard drugs. It was reported that in 2002, Genaera Corp. entered a 3-year option agreement with DuPont for its peptide intellectual property.

The third company is Intrabiotics Inc. located in Mountain View, USA (Hancock, 2000),(Zasloff, 2002),(Falla and Zhang, 2004),(Kues and Niemann, 2004),(Reddy *et al.*, 2004),(McPhee and Hancock, 2005). It focuses on the topical antimicrobial market, based on technology licensed from Bob Lehrer's laboratory at UCLA. The leading product is called iseganan (IB-367), an analogue of the porcine leukocyte peptide protegrin-1. Iseganan demonstrated a promising in vitro profile including wide spectrum antimicrobial spectrum with low resistance emergence and maintenance of activity in the saliva. With these promising properties, iseganan is intended for treatment of oral mucositis (an inflammation that occurs in patients who have undergone chemotherapy) and lung infections of CF patients that can lead to ventilator-acquired pneumonia (VAP). Phase I and II clinical trials showed promising results in treating *P. aeruginosa* infections, but in the phase III trials, higher rates of VAP and mortality in patients receiving iseganan caused the premature halting of the trial.

The next two companies, Migenix Inc. and Inimex Pharmaceutical Inc. are located in Vancouver, Canada. They focus on the commercialisation of peptides isolated from Bob Hancock's laboratory at the University of British Columbia (Hancock, 2000),(Zasloff, 2002),(Falla and Zhang, 2004),(Kues and Niemann, 2004),(Reddy *et al.*, 2004),(McPhee and Hancock, 2005),(Zhang & Falla, 2006). Migenix Inc., formerly known as Micrologix Biotech Inc., developed MBI-226 and MBI-594AN, products derived from indolicidin. MBI-226 is a topical antibiotic formulation to prevent sepsis

shock through reduction in central venous catheter (CVC) contamination. Colonisation of CVC by bacteria, which lead to sepsis shock of patients, is a growing problem in the USA. Phase III clinical trials of MBI-226 did not show a statistically significant drop in the rate of infection but it showed a significant decrease of the CVC colonisation. With these promising results, Migenix Inc. is collaborating with Cadence Pharmaceutical to carry out a phase IIIb clinical trial. The other product, MBI-594AN is also a topical drug used for acute acne treatment. A phase IIb clinical trial of MBI-594AN showed significant reduction of inflammatory acne with alcohol-based product containing 2.5% of MBI-594AN, indicating a future FDA application if it can pass the phase III clinical trial. The second company, Inimex Pharmaceutical Inc., focuses on development of peptides that are antimicrobial and that can boost host innate immunity. IMXC001, a short peptide with antimicrobial activity and immunostimulatory, is their promising product now undergoing pre-clinical trials.

1.8.2 Potential Applications for Ostrich Host Defence Peptides

With the current development of several host defence peptides for therapeutic products, ostrich host defence peptides also have a potential application for human therapeutic drugs. Therapeutic application of host defence peptides can be divided into pharmaceuticals or nutraceuticals (Anderson *et al.*, 2004). Pharmaceutical products, which are high value and highly pure, require complete clinical trials, whereas nutraceuticals are lower value products that do not require complete clinical trials or high purity. Furthermore, nutraceutical products can be commonly found in health food stores. Avian host defence peptides have shown a wide range in vitro activity against microorganisms, including anti-fungal activity against *Aspergillus fumigatus*. Even though not yet proven, avian peptides are believed to possess immune-enhancing functions such as those found in their mammalian counterparts. These functions of host defence peptides can add value to ostrich blood, which is currently waste material in the ostrich meat industries. A nutraceutical product is the most likely application of the ostrich host defence peptides. They can add value to the existing ostrich cream and oil that are commonly used as moisturisers and as a treatment for arthritis, eczema, burns, abrasions, sore joints, colitis and psoriasis. With the host defence peptides added to the cream/oil, these topical products can be used to protect wounds from bacterial or fungal infections and to enhance the immune response around the infected tissues.

Developments of ostrich host defence peptides into pharmaceutical products are also possible. Due to the high production cost, a candidate with great antimicrobial and immune-enhancing properties must firstly be sought. However, there will be a long period of time to develop this concept, since there has only been one peptide found from ostrich heterophil to date.

1.9 Conclusion

Animal and human immune systems can be divided into two types, innate immunity, which is activated by any invading pathogens, and adaptive immunity, which is a more specific defensive response than innate immunity and it is activated when the innate immunity is not capable of inactivating the pathogens. This innate immunity is found in all living organisms, whilst the adaptive immunity is only found in vertebrates. One component of the innate immunity is host defence peptides, which are relatively small molecules comprised of less than one hundred amino acids. These peptides are positively charged and they have an amphiphilic structure in solution. They have been isolated from granules of neutrophils in blood or epithelial cells of tissues. The primary function of these peptides is believed to be antimicrobial. In addition, these molecules have a significant role in inducing the innate and adaptive immune response of the host. The antimicrobial activity of host defence peptides is believed to be due to two different mechanisms. The first mechanism is known as the membrane disruptive mechanism, where the peptides disrupt the bacterial membrane, whereas the second mechanism is known as the non-membrane disruptive mechanism, where the peptides interact with bacterial intracellular components and inhibit the intracellular reaction of the bacterial cells.

One of the major host defence peptide families is defensins, which have been isolated from vertebrates, insects and plants. Vertebrate defensins exist in three different forms: α-, β-, and θ-defensins, of which only β-defensins have been found in mammalians, birds and humans, whereas the other two defensins are restricted to mammalians and humans. Vertebrate β-defensins can be further classified into three classes, based on the length and homology of the peptide and gene structures (Zhang *et al.*, 1998). The first class is mammalian and human β-defensins that contain short prepro-sequences (63-64 residues) and short introns (less than 1.6 kbp), the second class is β-defensins that

contain a long prepro-sequences (68-69 residues) with more than 6.5 kbp of introns and the third class is avian β-defensins.

The avian granulocytes lack peroxidases and alkaline phosphatases (Topp and Carlson, 1972),(Montali, 1988). As part of the non-oxidative mechanisms of the avian blood, the avian β-defensins are believed to play significant roles in avian immunity. Avian host defence peptides have been isolated from heterophils of chicken (gallinacin-1-2), turkey (THP-1 -3), ostrich (Osp-1), epithelial of chicken (gallinacins-3), turkey (gallopavin-1) and the stomach content of king penguins (sphenisein-1-2). The avian β-defensins have been extracted with acid extractions and purified using a combination of ion-exchange chromatography, gel filtration and RP-HPLC. Furthermore, there are gallinacins that have been identified from the chicken genome sequences. An evolutionary analysis showed that the avian β-defensins was related to mammalian β-defensins, suggesting that they might have originated from a common ancestral gene.

In addition, the literature review discussed the growing problems of antibiotic resistance that require developments of new classes of antimicrobial agents. In terms of application, the host defence peptides have been reported to undergo rapid developments in therapeutic industries. These developments have been based on investigations carried out by leading researchers. Some of the host defence peptides have undergone a phase III clinical trials but these peptides are limited by their low efficacy and safety. The potential application of ostrich host defence peptides from ostrich blood is considered to be essential in the development of novel nutraceutical products and pharmaceutical products.

1.10 Objectives of Research

The literature review on avian host defence peptides shows that very few studies have been undertaken in this field, especially the research on ostrich host defence peptides. Therefore, the objectives for this study are as follows:

1. To extract and purify host defence peptides from ostrich heterophils. The literature review indicated that avian heterophils contained several β-defensins. Therefore, it is believed that ostrich host defence peptides from heterophils will be mainly comprised of β-defensins.
2. To investigate the effect of different environmental factors, such as salts, cationic ions and temperatures, on the potency of ostrich host defence peptides. The presences of salt, cationic ions and temperatures are known to affect the activity of β-defensins.
3. To determine the mode of action of an ostrich host defence peptide by using one of the purified peptides. The mode of action of avian β-defensins is important to show how the peptides inhibit microorganisms. Since no studies of avian β-defensins have shown the mode of action, ostrich β-defensins would be compared with a human α-defensin and a sheep cathelicidin.

Chapter 2
Materials and Methods

2.1 Materials, Chemicals and Media

1) Fresh Blood

The main material was ostrich fresh blood that was obtained from Lamb/Venison Meat Packers Feilding Limited, Feilding, New Zealand.

2) Microorganisms

There were three types of organisms used commonly for antimicrobial activity tests with radial diffusion plate assay: *Escherichia coli* O157:H7, *Staphylococcus aureus* 1056MRSA (methicillin resistant *Staphylococcus aureus*) and *Candida albicans* 3153A. Each microorganism represents Gram-negative bacteria, Gram-positive bacteria and yeast, respectively. The *E. coli* was obtained from Communicable Disease Center, New Zealand, the *S. aurues* was obtained from Institute of Food Nutrition and Human Health, Massey University, New Zealand and the *C. albicans* was obtained from Institute of Molecular Biosciences, Massey University, New Zealand. These three types of microorganisms were expected to give an indication of the ostrich host defence peptides spectrum of activity. There were also other *Escherichia coli* strains used for a particular assay, which will be described in their respective sections.

3) Sodium Phosphate Buffer 100mM

A sodium phosphate buffer 100 mM (pH 7.4) was made by mixing a monobasic sodium phosphate and a dibasic sodium phosphate solution (Steinberg and Lehrer, 1997). The monobasic sodium phosphate solution was made by diluting 15.6 g of $NaH_2PO_4.2H_2O$ in 1 litre of water. The dibasic sodium phosphate solution was made by diluting 26.8 g of $Na_2HPO_4.7H_2O$ in 1 litre of water.

4) Underlay Agar

This agar was made by mixing 100 ml sodium phosphate buffer, 10 ml of TSB (Trypticase^{TM} Soy Broth; Difco 0370-17-3, Sparks, MD, USA) and 10 g of Ultra

Pure™ agarose (Invitrogen 15510-019, Carlsbad, CA, USA) (Steinberg and Lehrer, 1997). Deionised distilled water was added to bring the volume to 1 litre. The mixture was heated until the agarose dissolved and then 40 ml of aliquots was dispensed into 125 ml Durham bottles. Each bottle was sterilised and stored at room temperature. This agar contained a limited amount of nutrients and it was used for pathogens inoculations. The agar was liquefied and placed in a waterbath set at 50°C before use.

5) Overlay Agar

This agar was made by mixing 60 g of TSB and 10 g of agarose in 1 litre of deionised distilled water (Steinberg and Lehrer, 1997). The mixture solution was boiled until the agarose dissolved and then 40 ml of aliquots was dispensed into 125 ml Durham bottles. The bottles were then sterilised and stored at room temperature. This agar contained more TSB than the underlay agar, which are the nutrients used for growing the pathogens. The agar was liquefied and placed in a waterbath set at 50°C before use.

6) Synthesised Antimicrobial Peptide

There were two synthesised antimicrobial peptides: sheep myeloid antimicrobial peptides-29 (SMAP-29) and human neutrophil peptide-1 (HNP-1). SMAP-29 is a cathelicidin, an alpha-helical peptide that has 29 amino acid residues and a molecular weight of approximately 3179.99 Da (Mahoney *et al.*, 1995),(Tack *et al.*, 2002). This peptide was synthesised at Nucleic Acid Protein Service Unit (NAPS unit), University of British Columbia, Vancouver, Canada. HNP-1 is a beta-sheet peptide and belongs to a α-defensin family, which has a molecular weight of approximately 3439.51 Da (Ganz *et al.*, 1985),(Selsted *et al.*, 1985). This peptide was purchased from Peptide International Inc., Louisville, KY, USA. The amino acid sequences of SMAP-29 and HNP-1 are as follows:

- SMAP-29 NH_2-RGLRRLGRKIAHGVKKYGPTVLRIIRIAG
- HNP-1 NH_2-ACYCRIPACIAGERRYGTCIYQGRLWAFCC

7) 10X Binding Buffer for DNA Gel Electrophoresis

This buffer contains the following: 100 mM of Tris, 200 mM of KCl, 10 mM of EDTA, 10 mM of DTT and 50% (v/v) of glycerol (Park *et al.*, 1998). The pH was

adjusted with HCl to pH 8.0. The ingredient mixtures were autoclaved at 121°C for 15 minutes in order to avoid contamination, which could cause DNA degradation. Bovine serum albumin (BSA, Sigma-Aldrich A-7030, St. Louis, MO, USA) was added last, through a filter-sterilised syringe (Minisart 0.45 μm, Sartorius 16555 K, Hannover, Germany) to the final concentration of 500 μg/ml.

2.2 Peptide Extraction and Purification

2.2.1 Crude Extraction

The extraction method carried out was adapted from methods of purification of bovine neutrophil β-defensins (Selsted *et al.*, 1993). This method was modified to purify ostrich (Yu *et al.*, 2001) and ovine host defence peptides (Anderson and Yu, 2003).

The process began by collecting four litres of fresh blood in two containers stored in ice. Each container contained 200 ml of anti-coagulant 10% sodium citrate. The blood was then filtered to remove any feathers or other solid contaminants. An aqueous ammonium chloride 0.83% (w/v) concentration was mixed with the blood in 1:1 ratio to lyse the red blood cells. A mixture of lysed red blood cells and intact white blood cells was separated by centrifugation (Sorvall GS-3 rotor, 680g for 15 min, 4°C; Thermo Electron Corp., Waltham, MA, USA). The white blood cells were resuspended in PBSX buffer (13.7 mM of NaCl, 2.7 mM of KCl, 0.5 mM of $MgCl_2$ and 8.1 mM of Na_2HPO_4, pH 7.4). This solution was stained with Diff-Quick staining kit (Baxter Scientific B4132-1, Miami, FL, USA) and examined under the microscope. If red blood cells were still present, the red blood cells lysis step would be repeated.

A blender (MSE Atomix Blender) was then used for 5 minutes to disrupt the heterophils and to release the granules. These granules were collected with centrifugation (Sorvall SS-34 rotor, 27,000 g for 40 min, 4°C; Thermo Electron Corp., Waltham, MA, USA) and redissolved in 10% acetic acid. The solution was stained with the Diff-Quick staining kit in order to check the white blood cells. If the white blood cells were still present, the disruption step would be repeated.

The solution containing the granules was continuously stirred overnight at 4°C to extract the host defence peptides. After overnight extraction, the antimicrobial crude extract was separated from the heterophil granules with centrifugation (Sorvall SS-34

rotor, 27,000 g for 20 min, 4°C). Rota-evaporation (Buchi Rotavapor R110, Büchi Labortechnik AG, Flawil, Switzerland), with bath temperature set to approximately 37°C, was used to remove some acetic acid. The remaining acetic acid was removed with freeze-drying (Virtis freeze dryer model 10-020, Virtis, Gardiner, NY, USA). Finally, the extracted pellets were resuspended in 0.01% acetic acid. This solution, referred to the crude extract, would be used in the purification process.

2.2.2 Cation-exchange Chromatography

Cation exchange chromatography was used as the first purification step. The column was packed with Macro-Prep® CM, a weak cation exchange support, (Bio-Rad 156-0070, Hercules, CA, USA). After the crude extract was loaded into the column, the anionic components were eluted from the column using degassed 25 mM ammonium acetate solution. The bound cationic peptides were washed out with degassed 10% acetic acid. Both solutions were run at a flow rate of 20 ml/hr using a peristaltic pump (Amersham Biosciences Peristaltic Pump P-1, Castle Hill, NSW, Australia). A fraction collector (LKB Ultrorac fraction collector 7000, Bromma, Sweden) was used to collect the eluant in 10-minute fractions. A UV detector (LKB 2138 UVICORD S, Bromma, Sweden) and a chart recorder (Sekonic SS-250F recorder, Tokyo, Japan) were used to measure and record the absorbance over the time of the eluant. The chart recorder was set to a sensitivity of 0.1 and 30 mm/hr such that 5 mm on the chromatograph matched to one fraction. The fractions containing the anionic and cationic compounds from the column were collected separately in two containers. The 25 mM ammonium acetate buffer and the 10% acetic acid were removed using rota-evaporation and freeze-drying. Each fraction was then redissolved using 0.01% acetic acid. They were tested for antimicrobial activity (Chapter 2, Section 2.2.6) and the fraction showing antimicrobial activity was further purified with RP-HPLC.

2.2.3 RP-HPLC (Analytical Column)

The cationic components were readily separated into purified peptides using an analytical HPLC column; model Jupiter 4μ Proteo 90A, 250 mm x 4.6 mm, 4 micron (Phenomenex OOG-4396-EO, Torrance, CA, USA). The column was injected with 50

µl of the cationic fractions. The peptides were separated using gradient differences of two acetonitrile buffers: Buffer A contained 5% acetonitrile and 0.1% trifluoroacetic acid (TFA) and Buffer B contained 95% acetonitrile and 0.1% TFA. Elution of the antimicrobial peptides was carried out at 1 ml/min flow rate and monitored using a UV detector with wavelengths of 230 nm and 215 nm. Each purified peptide was collected in separate Eppendorf tubes, placed in a vacuum oven for one hour at 40°C and freeze-dried. They were tested for antimicrobial activity (Chapter 2, Section 2.2.6) and the fractions showing antimicrobial activity were characterised with mass spectrometry and N-terminal sequencing (Chapter 2, Section 2.2.4 and 2.2.5).

2.2.4 Mass Spectrometry

Mass spectrometry was carried out at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, Dunedin. The mass spectrometry was done using a MALDI-TOF-MS (matrix-assisted laser desorption/ionisation-time-of-flight-mass spectrometry). The samples were prepared by mixing peptide samples (0.5 µl, from 50 µl total volume HPLC fractions) and matrix (0.5 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% TFA, 60% acetronitrile) directly on a stainless steel slide. This solution was left to dry at room temperature. Mass data were collected at near threshold laser fluences in the positive ion mode, with a linear instrument (Finnigan Lasermat 2000, Thermo Bioanalysis, Hemel Hempstead, UK). For electrospray ionisation (ESI), the sample (diluted 1:1 from previous dilution in 50% MeCN) was directly infused at 3 µl/min into the Finnigan LCQ Deca Mass Spectrometer, using the Electrospray Ionisation Probe (Thermo Electron Corp., Waltham, MA, USA). Spray voltage was set to 5 kV and sheath gas (N_2) was set to approximately 33 units with capillary temperature set at 220°C. Data was collected over a series of scans and these were averaged to produce the spectrum observed.

2.2.5 N-Terminal Sequencing

N-terminal sequencing was also carried out at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, Dunedin. To analyse each peptide sequence, the procedure was carried out over a series of scans and these were averaged to produce the spectrum observed. Automated Edman peptide sequencing was

completed on a glass fibre disk using a Procise 492 Protein Sequencer (Applied Biosystems, Foster City, CA, USA), with pulsed liquid TFA delivery.

2.2.6 Radial Diffusion Plate Assay

This assay was used to test the antimicrobial activity (Steinberg & Lehrer, 1997). Three common pathogens used in the assay were *E. coli* O157:H7, *S. aureus* 1056MRSA and *C. albicans* 3153A. The pathogens were grown in TSB until they reached mid-log phase, which took approximately four hours. Each log culture was then diluted 10-fold and mixed with the underlay agar, poured into separate petri dishes and allowed to set. A number of wells were made in the agar to allow 5 µl addition of the test samples, which were the crude extract, cation exchange fractions and the purified peptides from RP-HPLC. A positive control and a negative control were always included. The positive controls were three antibiotics: 1 µg/ml polymyxin B sulphate (Sigma-Aldrich P-1004, St. Louis, MO, USA), 1 µg/ml Nisin (made from Nisaplin containing 2.5% nisin; Aplin and Barrett Ltd, Beaminster, England) and 10 µg/ml Nystatin (Sigma-Aldrich N-4503, St. Louis, MO, USA), which were antibiotics used for Gram-negative, Gram-positive and yeast, respectively. The negative control was 0.01% acetic acid. The plates were placed in a 37°C incubation room for an hour in order to allow the test samples to diffuse into the underlay agar. Overlay agar was poured onto the underlay agar and allowed to set before incubation overnight in a room at 37°C. On the following day, the antimicrobial activity of the tested samples was indicated by a clearing around the wells, which was measured with a ruler.

2.2.7 Protein Concentration Determination

Protein concentrations for the RP-HPLC peaks were determined using *dot*METRIC™ protein assay (Geno Technology Inc., St. Louis, MO, USA). The purified peptides were diluted with the supplied dilution buffer, which then pipetted onto a test strip. The strip was then placed in Fixer-A solution and incubated for 2 minutes at room temperature. After that, the strip was shaken in Developer-B solution for 30 seconds and incubated for 2-4 minutes to develop spots on the strips. These spots became visible within 2-3

minutes. They were compared to the *dot*METRIC™ scale to determine the protein concentration. This test was carried out in duplicate to ensure accuracy of results.

2.2.8 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of each purified peptides were determined using radial diffusion plate assays of diluted peptides (Lehrer *et al.*, 1991). The radial diffusion plate assay (Section 2.2.6) was carried out using multiple dilutions of the peptides, where protein concentrations have been determined using *dot*METRIC™ protein assay. After overnight incubation, the diameters of the clearing zone were measured with a ruler for each peptide concentration. The assay was carried out in duplicate. A graph of the size of the clearing on the plate, minus the size of the well, against log peptide concentration was plotted and a best-fit straight line was determined using regression. The MIC was then calculated by finding the intersection of the line with the x-axis, which indicated the peptide concentration required to have a zero clearing (Equation (1)). The average value of the duplicates was taken to give the final MIC values.

$$MIC = e^{\frac{-Y\,\mathrm{int}}{gradient}}$$ Equation (1)

where Y int and gradient are the y-intercepts and the constant gradient, respectively, obtained from the regression equation of the log peptide concentration graph.

2.3 Assessment of Peptide Activity in Variety of Conditions

2.3.1 RP-HPLC (Semi-prep column)

For characterisation of ostrich antimicrobial peptides, two ostrich peptides, namely ostricacin-1 and 2 (OSP), were chosen (Table 2.1). These two peptides were used for the study of the peptides antimicrobial activity under a variety of conditions and the investigation of mode of action of avian β-defensins against Gram-negative bacteria. Both peptides were not commercially synthesised due to high cost. It has been reported that synthesising defensins is difficult and expensive, thus, limiting their use for investigation into mode of actions (Boman, 2003). This outcome is believed to be due to the three-disulphide bridges, which are formed by the six cysteines in a specific manner.

Therefore, to generate sufficient materials for the characterisation tests, a semi-prep HPLC column, model Jupiter 10μ Proteo 90A, 250 mm x 10 mm, 10 micron (Phenomenex OOG-4397-NO, Torrance, CA, USA) was used. With the semi-prep column, the injection volume was increased 4-fold to 200 μl. The peptides were separated using gradient differences of the same acetonitrile buffers. Elution of the antimicrobial peptides was carried out at 4 ml/min flow rate and monitored using the same UV detector with wavelength of 230 nm and 215 nm. The purified peptides were collected, placed in a vacuum oven for one hour at 40°C and freeze-dried.

Table 2.1 Sequence and Molecular Mass of the Ostrich Antimicrobial Peptide, Osp-1 and 2. The residues in red bold indicate the β-defensin core motif.

Name	Molecular Mass	Amino Acid Sequence
Osp-1	4009.8	-----LFCR--KGTCHFGGCPAHLVKVGSCFGFRACCKWPWDV
Osp-2	4704.9	APGNKAECEREKGYCGFLKCSFPFVVSGKCSRFFFCCKNIW–
	Consensus	-------**C---KG-C-F--C----V-G-C--F--CCK--W--**

2.3.2 MIC using Micro Broth Dilution

A modified broth dilution method with Mueller-Hinton broth in polypropylene microtiter plates was used as an alternative method in order to determine the MIC of Osp-1 and Osp-2 (Wu and Hancock, 1999a),(Zhang *et al.*, 2000)

Each pathogen, *E. coli* O157:H7 and *S. aureus* 1056MRSA, was grown at 30°C in Mueller-Hinton Broth (MHB, Merck 110293, Darmstadt, Germany) to OD_{600} of 0.1. This culture was then diluted down 1000-fold. A media containing 0.2% BSA and 0.01% acetic acid was added to each well of the microtiter plate using a multipipettor set at 50 μl, except for the first column. The same media was used to dilute the peptides to 2X of the desired concentration. The first column was added with 50 μl of the peptide with 2X concentration. A series of two-fold dilutions were made across to the tenth column using a multipipettor set at 50 μl. The diluted microorganisms were then added last, starting from the last column that contained peptides with the lowest concentration. The two peptides were tested separately on different microtiter plates. The plates were incubated at 37°C overnight. The MIC values were determined at the lowest

concentration at which the growth was inhibited, indicated by the absence of precipitates. The assays were carried out in duplicate.

However, this method appeared to require high concentration of peptides, which could not be produced with the semi-prep RP-HPLC column. Therefore, the radial diffusion plate assay method was used, instead of this micro broth dilution method. The radial diffusion plate assay method was not suitable for an investigation into changes in pH, because the pH of the agar was changed after sterilisation, hence, the change in pH was not included in this study.

2.3.3 Monovalent cation Effects

The effect of monovalent cation concentration on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides against *E. coli* O157:H7 and *S. aureus* 1056MRSA, in a variety of monovalent cation concentrations. A modified radial diffusion plate assay, described in Section 2.2.6, was used. There were two monovalent cations (Na^+ and K^+) used in this assay in the form of chloride salts. NaCl or KCl was added to the underlay agar with the final concentrations of 0, 10, 50, 100, 200 and 500 mM. The assays were independently carried out three times with duplicates on every run. The average, standard deviations and 95% confidence intervals of the MICs were determined using "AVERAGE", "STDEV" and "CONFIDENCE" functions in Microsoft Excel. The average MIC values and the error limit, which was calculated adding and subtracting the confidence interval value to the average value, are plotted for each cationic ion concentration as histograms.

2.3.4 Divalent Cation Effects

The effect of divalent cation concentration on the antimicrobial activity of the peptides was tested by determining the MICs of the peptides against *E. coli* O157:H7 and *S. aureus* 1056MRSA, in a variety of divalent cation concentrations. This was accomplished in the same way as described in Section 2.3.3 for determining the effect of the monovalent cation concentrations on the activity of the peptides. There were two divalent cations (Ca^{2+} and Mg^{2+}) used in this assay. They were added to the underlay agar as chloride salts. The final concentrations of these divalent cations in the underlay

agar were 0, 2, 5 and 10 mM. The assays were independently carried out three times with duplicates on every run. The average, standard deviations and 95% confidence intervals of the MICs were determined using "AVERAGE", "STDEV" and "CONFIDENCE" functions in Microsoft Excel. The average MIC values and the error limit, which was calculated adding and subtracting the confidence interval value to the average value, are plotted for each cationic ion concentration as histograms.

2.3.5 Temperature Effects

The effects of temperature on the antimicrobial activity of the peptides were tested by determining the MIC of the peptides against *E. coli* O157:H7 and *S. aureus* 1056MRSA, after they had been heated to a variety of temperatures. The MICs were determined using the radial diffusion plate assay method, as described in Section 2.2.6. Before the MIC assay, the peptides were heated for 30 minutes and then cooled on ice. The temperatures, to which the peptides were heated, were 30, 50, 70 and 90°C. There were also peptides that were autoclaved (121°C, 20 minutes). The average, standard deviations and 95% confidence intervals of the MICs were determined using "AVERAGE", "STDEV" and "CONFIDENCE" functions in Microsoft Excel. The average MIC values and the error limit, which was calculated adding and subtracting the confidence interval value to the average value, are plotted for each cationic ion concentration as histograms.

2.3.6 Analysis of Variance (ANOVA)

Analysis of variance (ANOVA) of the MIC data from each experiment were analysed using MINITAB program in order to determine if there was a difference between the mean MICs for the different peptides and for the different conditions. The "two way ANOVA" function with confidence intervals (CI) of 95% was used. The y-variate was ln MIC of the peptide, whilst the row and column factors were the concentration of the cations/temperature conditions and the number of repeats, respectively. The p-value, indicated in the last column, showed whether the environment factors significantly affected the antimicrobial activity of the Osp-1 and 2 (i.e. p value is less than 5%).

2.4 Study of Antimicrobial Peptide Mechanisms of Action

2.4.1 LPS Binding Assay

Lipopolysaccharide (LPS) is part of the outer cell wall of Gram-negative bacteria. It is believed that the cationic antimicrobial peptides bind to negative charged lipopolysaccharide (LPS), due to the electrostatic interaction. The ability of ostricacins to bind to LPS was determined by determining their ability to displace bound fluorescent dye, dansyl polymyxin B (DPX) (Moore *et al.*, 1986),(Hancock *et al.*, 1991),(Anderson *et al.*, 2004),(Powers *et al.*, 2004)

The LPS isolated from *Escherichia coli* O111:B4 (Sigma-Aldrich L-2630, St. Louis, MO, USA) was resuspended in 5 mM HEPES buffer pH 7.2 (Sigma-Aldrich H-3375, St. Louis, MO, USA), to a concentration of 3 μg/ml. For the assay, 2 mL of the LPS solution were mixed with 20 μl of 100 μM DPX (Invitrogen P-13238, Carlsbad, CA, USA) in a 1-cm quartz cuvette, at which the LPS would be saturated with DPX.

The fluorescence intensity was measured using a spectrofluorophotometer (Shimadzu Corp. model RF-1501, Kyoto, Japan) with an excitation and an emission wavelength set at 340 nm and 485 nm, respectively. After the initial reading was recorded, the mixture was titrated with an equal amount of the test peptides. The fluorescence reading was recorded after each peptide addition. From the collected data, the fraction of fluorescence inhibited was calculated using Equation (2).

$$F_{inhib} = \frac{F_{initial} - F_n}{F_{initial}} \qquad \text{Equation (2)}$$

where F_{inhib} is the fraction of fluorescence inhibited, $F_{initial}$ is the initial fluorescence before any peptides addition and F_n is fluorescence reading at concentration n.

The fraction of fluorescence inhibited was then plotted against the peptide concentrations. If the test peptides were able to displace some DPX from the LPS, the fluorescence intensity would decrease, hence the fraction of fluorescence inhibited would increase. SMAP-29 and HNP-1 were used for comparison with Osp-1 and 2.

2.4.2 Outer Membrane Permeabilisation Assay

This assay was carried out at The Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada. In this assay, the ability of the antimicrobial peptide to lyse bacterial outer membrane was carried out by assessing their ability to promote uptake of 1-*N*-phenylnapthylamine (NPN) (Loh *et al.*, 1984),(Hancock *et al.*, 1991),(Powers *et al.*, 2004). If the peptide is able to promote uptake of the NPN, the fluorescent dye will illuminate inside the bacterial cytoplasm.

A log culture of *E. coli* UB1005 was grown in Luria-Bertani (LB) Broth (Difco 244620, Sparks, MD, USA) at 30°C until an OD_{600} of 0.5. This culture, *E. coli* UB1005, is a wild type strain of *E. coli* obtained from the University of British Columbia, Vancouver, Canada. The cells were then centrifuged (Sorvall SS-34, 10,000 rpm for 5 min, room temperature) and washed in 5 mM HEPES, 5 mM glucose buffer pH 7.4 containing 5 μM carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP, Sigma-Aldrich C2759, St. Louis, MO, USA). The cells were collected with Sorvall SS-34, 18,000 g for 5 minutes at room temperature and re-suspended in the corresponding buffer to the same optical density.

The fluorescence intensity was measured, using a model LS50B luminescence spectrometer (Perkin Elmer, Shelton, CT, USA) with an excitation and an emission wavelength set at 350 nm and 420 nm, respectively. A 2 ml cell suspension was placed in a 1 cm cuvette and mixed with 20 μl of 0.5 mM NPN (Invitrogen P-65, Carlsbad, CA, USA). The background fluorescence of the cell suspension was monitored for 30 seconds before the addition of NPN, to ensure background fluorescence was nil and an additional 60 seconds was monitored to ensure cell stability. The peptide was then added to a final concentration of 1 μM or 0.5 μM in the case of polymyxin B sulphate, the positive control. Changes in fluorescence intensity were recorded until a plateau was observed. When the plateau appeared, it indicated that maximum uptake of the NPN dye was reached and the assay was stopped. SMAP-29 and HNP-1 were used for comparison with OSP-1 and 2.

2.4.3 Cytoplasmic Membrane Depolarisation Assay

This assay was also carried out at The Centre for Microbial Diseases and Immunity Research, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada. In this assay, cytoplasmic membrane depolarisation ability was determined using a membrane potential sensitive dye, 3,3-dipropylthiacarbocyanine iodide ($diSC_3 5$), and *Escherichia coli* DC2, previously described in (Wu and Hancock, 1999a),(Wu *et al.*, 1999b),(Zhang *et al.*, 2000),(Powers *et al.*, 2004).

The log culture of *E. coli* DC2 strain was grown in Luria-Bertani (LB) Broth at 30°C until an OD_{600} of 0.5. *E. coli* DC2 is an outer membrane permeable mutant obtained from the University of British Columbia, Vancouver, Canada. The cells were centrifuged (Silencer, 3000 rpm for 10 min, room temperature) washed and resuspended in 5 mM HEPES, 5 mM glucose buffer pH 7.4 to an OD_{600} of 0.05. A $diSC_3 5$ (Invitrogen D-306, Carlsbad, CA, USA) stock solution was added to a final concentration of 0.4 μM and the cell suspension was incubated at room temperature for 30 minutes to allow quenching. KCl solution was then added to the cell mixture to a final concentration of 0.1 M and incubated at room temperature for another 10 minutes to equilibrate the cytoplasmic and external K^+ concentration.

A 2-ml of the suspension, containing the cells, $diSC_3 5$ and KCl, was placed in a 1-cm cuvette and measured for its initial fluorescence, using a model LS50B luminescence spectrometer (Perkin Elmer, Shelton, CT, USA) with an excitation and an emission wavelength set to 622 nm and 670 nm, respectively. The peptide was then added to the mixture to a final concentration of 1 μM. In all cases, the background fluorescence of the cell suspension was monitored for 60 seconds before the addition of the peptides to ensure cell stability. Changes in fluorescence were monitored until a plateau was reached. SMAP-29 and HNP-1 were used for comparison with OSP-1 and 2. Gramicidin (Sigma-Aldrich G-5002, St. Louis, MO, USA) was used as a positive control.

2.4.4 Optical Density and Colony Forming Unit Measurements

Measurements of optical density (OD) and colony formation units (CFU) were carried out over time to determine whether the antimicrobial peptides could kill or inhibit growth of *E. coli* O111. This *E. coli* culture was obtained from Communicable Disease Center, New Zealand. The Gram-negative bacterium was grown overnight at 30°C in MHB, which was then used to inoculate the log culture. After 2 hours incubation, the log culture was diluted to OD_{600} of 0.1 and it was then placed into four cuvettes. These cuvettes contained OSP-1, OSP-2, polymyxin B sulphate (positive control) and 0.01% acetic acid (negative control), respectively. At time zero, 100 µl of the peptides or 20 µl of the controls were added to their respective cuvettes and recorded for initial OD_{600}. The absorbance was then recorded every 15 minutes for the first hour and then at 1.5 hrs and at 2 hrs, using a double beam spectrophotometer (Hitachi U-2000, Tokyo, Japan). Each time the OD_{600} was recorded, a 100 µl of the culture was taken to make serial dilutions up to 10^{-7} using peptone water (Merck, Darmstadt, Germany). For the 10^{-5}-10^{-7} dilution, 1 ml was taken out for plating on a plate count agar (Merck, Darmstadt, Germany) and incubated overnight at 37°C. During the OD measurements, the cuvettes were incubated in a waterbath at 37°C. After overnight incubation, plates containing the appropriate dilutions were counted for CFU, using a colony counter (Suntex model CC-560, Taipei County, Taiwan).

2.4.5 DNA Gel Electrophoresis

To determine whether the ostrich peptides were able to bind the DNA, a DNA gel electrophoresis was carried out (Park *et al.*, 1998). The bacterial DNA used for this test was λDNA/*Eco*RI marker (Promega US G1721, Madison WI, USA). The DNA was mixed with 10X binding buffer and the peptides (OSP-1 and OSP-2) according to the ratio shown in Table 2.2. Oabac5mini was included for a control because this peptide was known to bind bacterial DNA (Anderson, R.C., unpublished data). Water was added to each solution up to a final volume of 20 µl. The solutions were incubated at room temperature for an hour.

To trace the DNA, 2 µl of loading buffer (20% (w/v) sucrose and 0.125% (w/v) bromophenol blue) were added before they were loaded onto a 0.75% agarose/TAE gel. The gel was run at 108 V and 70 mA for approximately 1.5 hours with a running buffer

(0.04 M of Tris and 0.001 M of EDTA, pH 8.0). The gel was then stained with ethidium bromide (Boehringer Mannheim Gmbh, Mannheim, Germany) and photographed with Gel Doc XR System (Bio-Rad Laboratories, Hercules, CA, USA).

Table 2.2 Ingredients for Peptide-DNA Binding Gel Electrophoresis.

	Volume Required (μl)					
DNA:Peptide	**1:0**	**1:0.5**	**1:1**	**1:2**	**1:4**	**1:8**
Eco RI (0.1μg /μl)	1	1	1	1	1	1
10x Binding Buffer	2	2	2	2	2	2
Peptides	0	1	2	4	8	16

Chapter 3

Extraction and Purification of Host Defence Peptides from Ostrich Heterophils

3.1 Introduction

In Chapter 1, it was explained that host defence peptides isolated, from avian heterophils, were merely β-defensins (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Yu *et al.*, 2001). The β-defensins are a sub-family of defensins, which is a family of host defence peptides characterised by triple-stranded antiparallel β-sheet structures and three intramolecular disulfide bridges formed by six cysteine residues (White *et al.*, 1995),(Harmon, 1998),(Lehrer and Ganz, 2002),(Ganz, 2003),(Torres and Kuchel, 2004). In addition to the host defence peptides, there are also peptides with β-defensin-like-folding found in the toxin of rattlesnake, sea anemone and platypus. They are believed to represent an adaptation of epithelial host defence peptides for efficacy against larger predators.

Our interest in research into antimicrobial peptides from ostrich blood was two-fold. Firstly, since avian species rely very much on non-oxidative mechanisms, composed of host defence peptides and lysozyme, the presence of these cationic peptides in ostriches indicates that these peptides are the primary mechanism for their survival as one of the oldest order of flightless birds (Evans *et al.*, 1994),(Harmon, 1998),(Yu *et al.*, 2001). In addition, there has been very limited research on avian host defence peptides, as described in Chapter 1. Secondly, these peptides can be developed to increase the value of ostrich blood, which is currently discarded as waste effluent in New Zealand meat processing plants. They can be developed into nutraceutical products, such as a crude extract that contains a mixture of ostrich antimicrobial peptides and ostrich topical products that has antimicrobial properties. With additional antimicrobial properties, these peptides can add more value to New Zealand ostrich industries.

Previous research into the purification of ostrich host defence peptides has been undertaken at Massey University (Yu *et al.*, 2001),(Tan and Yu, 2002, unpublished

results). Yu *et al.* (2001) successfully isolated one ostrich host defence peptide called ostricacin-1 (Osp-1). Osp-1 appeared to be β-defensins that showed activity against *E. coli* O111 and *S. aureus* NCTC4163, whilst Tan and Yu (2002) identified ostricacin-1 and another peptide with activity against *E. coli* O157:H7, *S. aureus* 1056MRSA and *Candida albicans* 3153A. However, Tan and Yu (2002) could not characterise the peptide, due to poor separation in the RP-HPLC column.

The main objective of this part of research was to isolate and purify host defence peptides components from ostrich heterophils. Once purified, a radial diffusion plate assay method was used in order to test the antimicrobial activity of the ostrich peptides against *E. coli* O157:H7, *S. aureus* 1056MRSA and *Candida albicans* 3153A. The *E. coli* and *C. albicans* are well-known pathogen strains, whereas the *S. aureus* is a Gram-positive resistant bacterium that causes infections ranging from skins abscesses to life-threatening conditions, such as endocarditis and toxic shock. The molecular weights and N-terminal sequences were then determined and compared with the other β-defensins. From previous investigations of avian host defence peptide, it could be hypothesised that the peptides would be β-defensins.

3.2 Results and Discussion

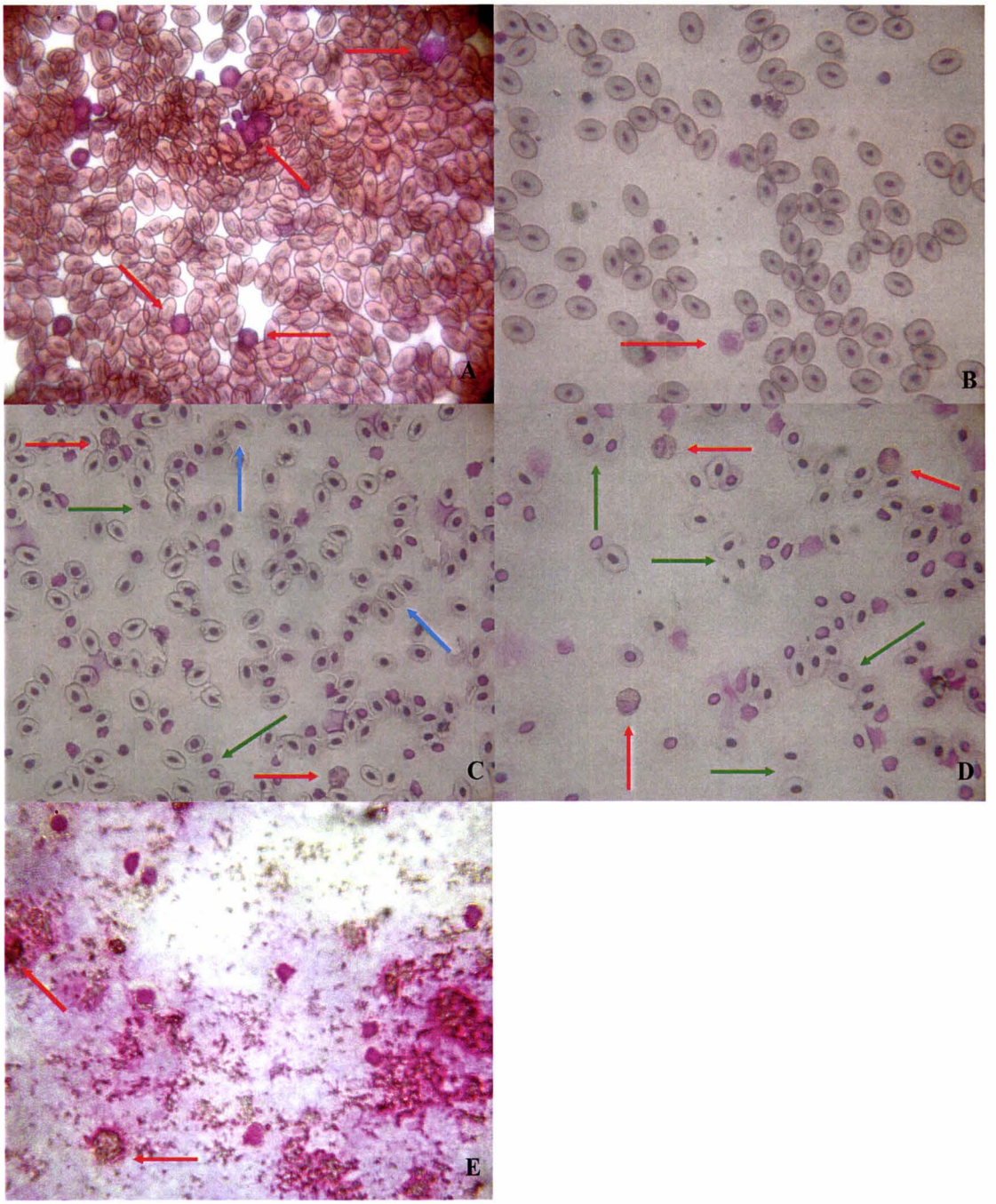
3.2.1 Peptide Extraction

Extraction of host defence peptides from ostrich heterophils could be divided into three stages: disruption of red blood cells through isotonic shock; disruption of the heterophils (granulocytes); and extraction of the host defence peptides from the granules. These three stages are outlined in Chapter 2, Section 2.2.1. Each stage of extraction was followed by a centrifugation step that removed the unwanted materials and a microscopic examination of blood samples, which had been stained with a Diff-Quick staining kit. The microscopic examination was carried out to ensure that each stage was completed (Figure 3.1).

The isotonic shocks had to be repeated three times in order to disrupt most of the red blood cells. After centrifugation, the heterophils were resuspended in a PBSX buffer and disrupted using a blender. The granules were separated with a centrifuge and resuspended in 10% acetic acid. Extraction of the host defence peptides was carried out overnight. The crude extract was then freeze-dried and resuspended in 0.01% acetic acid. Antimicrobial activity, with radial diffusion plate assay, showed that the crude extract had activity against *E. coli* O157:H7, *S. aureus* 1056MRSA and *C. albicans* 3153A (Table 3.1). The positive controls were: Polymyxin B Sulphate, Nisin and Nystatin, which were used for *E. coli* O157:H7, *S. aureus* 1056MRSA and *C. albicans* 3153A, respectively. The negative control was 0.01% acetic acid. To purify and isolate the host defence peptides, the crude extract was then run through a cationic exchange column, followed by purification through a RP-HPLC column.

In this extraction method, two modifications were made to reduce the time-consuming extraction process previously reported (Yu *et al.*, 2001), (Anderson and Yu, 2003). The first modification was a change of ratio of ammonium chloride to blood cells, from 3:1 to 1:1. The change in ratio reduced the working volume and hence it reduced the extraction time. The second modification was in the disruption of the granulocytes step, where a blender replaced the sonicator. Sonicators are known to generate more heat than blenders and the increase of temperature is not desirable, because it can affect the peptides potency. During the course of work in 2003, Anderson and Yu found that a blender worked as efficiently as a sonicator. Therefore, in our research, the blender was preferred instead of the sonicator.

Figure 3.1 Microscopic Examination (400x) of Blood Cells Stained with Diff-Quick. The ostrich blood comprised of red blood cells and heterophils (➔) (A). The number of red blood cells decreased, whilst the heterophils (➔) remained intact, after the first isotonic shock (B). After the second isotonic shock, there were some intact red blood cells (➔) and lysed red blood cells (➔) (C). After the third isotonic shock, most red blood cells were lysed (➔), whilst the heterophils (➔) remained intact (D). Lastly, (E) shows the heterophils were successfully disrupted (➔).



3.2.2 Peptide Purification with Cationic Exchange

The crude extract was passed through the cationic exchange column, which was packed with Macro-prep® CM, in order to separate the cationic components from the anionic components. This method is described in Chapter 2, Section 2.2.2. A typical chromatograph, representing a cationic exchange chromatography run, is shown in Figure 3.2. The non-cationic components (F1) were eluted with 25 mM ammonium acetate solution, whilst the cationic components (F2) were retained in the column and washed out with 10% acetic acid. Antimicrobial activity tests of the cationic and anionic fractions showed that the activity of crude extract was mainly retained in the cationic fraction (Table 3.1). The positive controls were Polymyxin B Sulphate, Nisin and Nystatin, which were used for *E. coli* O157:H7, *S. aureus* 1056MRSA and *C. albicans* 3153A, respectively. The negative control was 0.01% acetic acid. The two fractions were divided into several aliquots and kept in freeze-dried form to avoid peptide degradation. Each aliquot was resuspended and then ran through an RP-HPLC column.

Figure 3.2 Ion-Exchange Chromatograph of the Ostrich Crude Extract. The column was run initially with 25mM ammonium acetate. At 'elute', the running buffer was changed to 10% acetic acid and at 'Wash' the running buffer was changed to 20% ethanol.

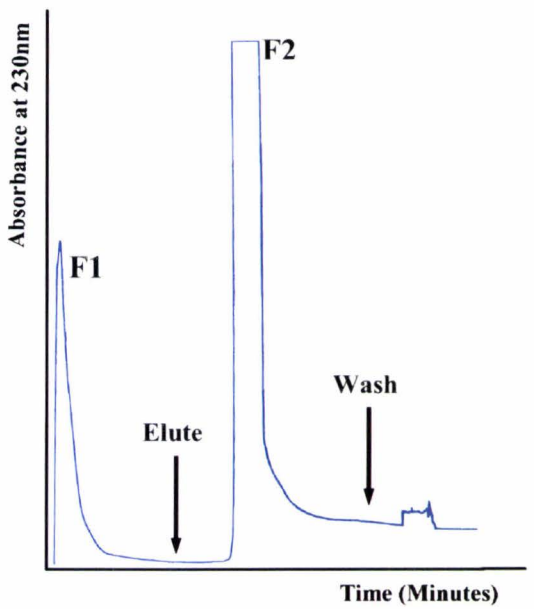


Table 3.1 Plate Assay of Ion Exchange Column Fractions and Crude Extract.

Tested Sample	Clearing Diameters (mm)		
	E. coli O157:H7	*S. aureus* 1056MRSA	*C. albicans* 3153A
Crude extract	7	8.5	8
Fraction 1	No clearing	No clearing	No clearing
Fraction 2	7	8	6
Positive	10	8	8
Negative	No clearing	No clearing	No clearing

3.2.3 Peptide Purification with RP-HPLC (Analytical Column)

In the RP-HPLC column, the cationic fraction was readily separated into 24 peaks when the column was run with a gradient of 21-29% acetonitrile containing 0.01% TFA (Figure 3.3). This method is described in Chapter 2, Section 2.2.3. These 24 peaks were then tested for antimicrobial activity against the three pathogens (Table 3.2). All peaks showed activity against the bacteria, except for Peak 6, 7, 21 and 22. Peak 5, 9, 13, 14, 19 and 20 were chosen for characterisation, using mass spectrometry and N-terminal sequencing. Peak 8, 11, and 15-17, which also showed activity, were not characterised, because they were not well separated. Peak 24, which appeared at the washing phase, also showed activity, indicating a possibility of more host defence peptides present in a higher acetonitrile gradient. Purification of these other peptides was attempted using a gradient of 29-36% acetonitrile containing 0.01% TFA. This attempt was not successful, because the peptides were not well separated (results not shown).

Figure 3.3 RP-HPLC chromatograph of Fraction 2 of the Cationic Exchange Column. The red arrows indicate peaks that were selected for further analysis.

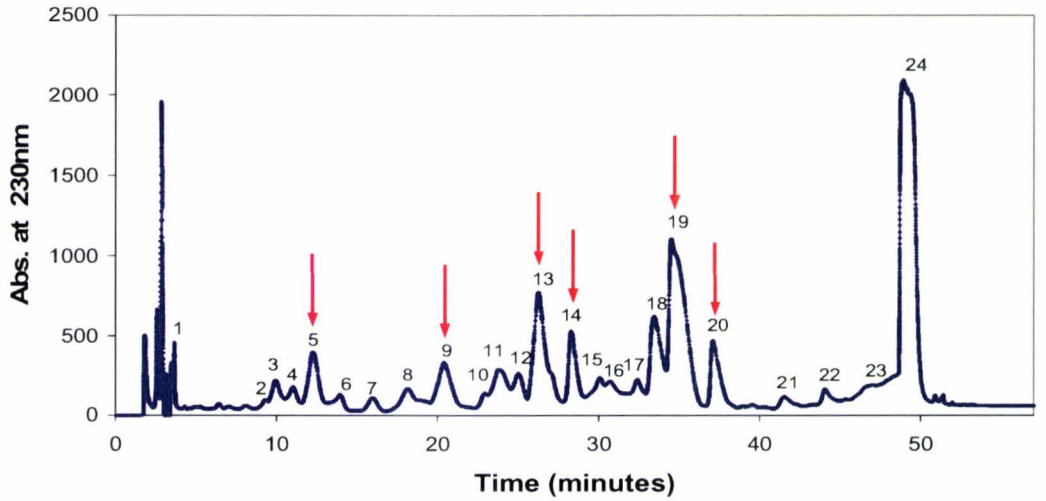


Table 3.2 Plate Assay of RP-HPLC Peaks.

Tested Sample	Clearing Diameters (mm)		
	E. coli O157:H7	*S. aureus* 1056 MRSA	*C. albicans* 3153A
Peak 1-4	2	1.5	No Clearing
Peak 5	2	2	No Clearing
Peak 6-7	No Clearing	No Clearing	No Clearing
Peak 8	3	3	No Clearing
Peak 9	3	3	No Clearing
Peak 10	2	2	No Clearing
Peak 11	4	5	3.5
Peak 12	2	3	1
Peak 13	3.5	4	3
Peak 14	3	2.5	1
Peak 15-17	3	3	No Clearing
Peak 18	3.5	3.5	1
Peak 19	4	4	2
Peak 20	3.5	3.5	2
Peak 21-22	No Clearing	No Clearing	No Clearing
Peak 23	2	2	No Clearing
Peak 24	4	4	2

3.2.4 Mass Spectrometry and N-terminal sequence Analysis

The molecular weights of the six RP-HPLC peaks were determined using MALDI-TOF mass spectrometry. This method is described in Chapter 2, Section 2.2.4 and 2.2.5. The molecular weights of Peak 5, 9, 19 and 20 were determined to be 4979.1, 4752.4, 4009.8 and 4704.9 Da, respectively (Table 3.3). The amino acid sequences of these four peptides were then determined with N-terminal sequencing analysis (Table 3.3). From the mass spectrometry and N-terminal sequencing, Peak 19 was confirmed to be Osp-1. The N-terminal sequence analysis of subsequent Peak 20, 5 and 9 revealed that these peptides were comprised of 36-42 amino acid residues. Peak 20, 5 and 9 were named as ostricacin-2, 3 and 4 (Osp-2-4), respectively. The other two peaks, Peak 13 and 14, appeared to comprise a mixture peptide (Protein Microchemistry Facility, personal correspondence, 2004). The MALDI-TOF of Peak 13 showed a major peak at approximately 4.2 kDa and a minor peak at 5.36 kDa, whilst Peak 14 had a major peak at 5.36 kDa and two minor peaks at 3.6 kDa and 3.9 kDa. Therefore, the N-terminal sequencing of Peak 13 and 14 were not carried out.

The four ostricacins can be classified as β-defensins, since their molecular weights and their amino acid residues are within the range of those known β-defensins (molecular weight of β-defensins is between 4-6 kDa with amino acid residues between 36-42 residues (White *et al.*, 1995),(Ganz, 1999),(Lehrer and Ganz, 2002),(Torres and Kuchel, 2004)).They share eight conserved residues that include the six-cysteine residues and two glycines, Gly^{13} and Gly^{28} (indicated as overall consensus in Table 3.3). As β-defensins, the intramolecular disulfide bonds are formed through the pairing of $cysteine^{8}$-$cysteine^{38}$, $cysteine^{15}$-$cysteine^{31}$, and $cysteine^{20}$-$cysteine^{39}$. These cysteine residues are known as the stabiliser of β-defensins molecules. Harwig *et al.* (1994) suggested that the six-cysteines, Gly^{13}, Pro^{21} and Gly^{28} were essential structural elements of the β-defensins and they were designated as 'β-defensin core motif' (Harwig *et al.*, 1994). However, $proline^{21}$ is not commonly conserved in other β-defensins, as shown in Table 3.3. Therefore, the 'β-defensin core motif' is proposed to comprise of only eight residues instead of nine.

Table 3.3 Mass spectrometry and N-terminal sequencing of Osp-1-4. Using FASTA, these ostricacins were aligned with the chicken gallinacins and turkey THPs.

Peptides	Complete sequence	Mol. Weight	Similarity
	1 10 20 30 40 45		
Osp-1 (P19)	-----LF**C**--RK**G**T**C**HFGG**C**-PAHLVKV**G**S**C**FGFR-A**CC**KWPWD	4009.8	
THP-2	-----LF**C**--KR**G**T**C**HFGR**C**-PSHLIKV**G**S**C**FGFR-S**CC**KWPWD	4063.8	82.9%
Gal-2	-----LF**C**--KG**G**S**C**HFGG**C**-PSHLIKV**G**S**C**FGFR-S**CC**KWPWN	3916.0	80.0%
Consensus	-----LF**C**--+-**G+C**HFG-**C**-P+HL+KV**G**S**C**FGFR-+**CC**KWPW+		
Osp-2 (P20)	APGNKAE**C**EREK**G**Y**C**GFLK**C**-SFPFVVS**G**K**C**SRFF-F**CC**KNIW-	4704.9	
Gal-1	--GRKSD**C**FRKS**G**F**C**AFLK**C**-PSLTLIS**G**K**C**SRFY-L**CC**KRIWG	4505.0	56.4%
Gal-1α	--GRKSD**C**FRKN**G**F**C**AFLK**C**-PYLTLIS**G**K**C**SRFH-L**CC**KRIW-	4582.0	56.4%
THP-1	--GKREK**C**LRRN**G**F**C**AFLK**C**-PTLSVIS**G**T**C**SRFQ-V**CC**KTLLG	4425.3	50.0%
Consensus	--G:+-:**C**-R::**G+C**:FLK**C**-:---++S**G**-**C**SRF---**CC**K-+--		
Osp-3 (P5)	IPRPLDP**C**IAQN**G**R**C**FTGI**C**-RYPYFWI**G**T**C**RNG-KS**CC**RR--R-	4979.1	
Gal-7	IPRPIDT**C**RLRN**G**I**C**FPGI**C**-RRPYYWI**G**T**C**NNGIGS**CC**ARGWRS	5003.8	67.5%
Consensus	IPRP+D:**C**--+N**G**-**C**F:GI**C**-R-PY+WI**G**T**C**:NG--S**CC**-R--R-		
Osp-4 (P9)	LPVNEAQ**C**RQVG**G**Y**C**GLRI**C**-NFPSRFL**G**L**C**TRNH-P**CC**SRVWV	4752.4	
Gal-8	--NNEAQ**C**EQAG**G**I**C**SKDH**C**FHLHTRAF**G**H**C**QRGV-P**CC**RTVYD-	4593.1	46.2%
Consensus	---NEAQ**C**:Q:G**G**-**C**:---**C**-++-+R-+**G**-**C**-R:--P**CC**--V+--		
Overall Consensus	-------C----G-C----C--------G-C------CC------		

Note: "–" indicates no identical or conserved residues have been observed; "+" indicates conserved substitutions have been observed according to their hydrophobicity or charge; and ":" indicates semi-conserved substitutions are observed according to their hydrophobicity or charge. Molecular weights and sequence of Gal-1, -1α, -2 and the Osps were based on mass spectrometry measurement and sequence determination of the isolated peptides, whilst Gal-7, Gal-8, THP-1 and THP-2 were based on genomic data (Evans *et al.*, 1994),(Harwig *et al.*, 1994),(Xiao *et al.*, 2004)

A database search using FASTA was then carried out, based on the amino acid sequence of Osp-1-4, in order to identify relationships between these ostricacins with other β-defensins. The results, also displayed in Table 3.3, show that the closest relations to Osp-1, 2, 3 and 4 are two turkey β-defensins (THP-1 and 2) (Evans *et al.*,

1994) and five chicken gallinacins (Gal-1, 1α, 2, 7 and 8) (Harwig *et al.*, 1994),(Xiao *et al.*, 2004).

The highest percentage similarities are shown between Osp-1 and THP-2 (82.9%) with 29 identical amino acid residues and between Osp-1 and Gal-2 (80%) with 27 identical amino acid residues. These three peptides, Osp-1, Gal-2 and THP-2 share 27 conserved residues. Compared with the other avian β-defensins, these three peptides have some residues truncated at the amino termini. They also have a unique characteristic with two missing residues between cysteine[8] and cysteine[15]. In addition, these peptides' sequences always show leu-phe-cys motif at the amino termini and lys-trp-pro-trp-asn/asp motif at the carboxyl termini.

The other ostricacins show only moderate similarity to their chicken and turkey counterparts. Furthermore, each ostricacins and their avian counterparts retain a distinctive motif at the amino termini. Osp-2 showed 56.4% similarity to Gal-1 and Gal-1α and 50% similarity to THP-1. These four peptides share 18 conserved residues. They have a distinct motif of five residues at the amino terminus, which is comprised of glycines, two basic residues (lysine or arginine) and two varying residues, before cysteine[8]. The two varying residues are usually polar with charge residues, except Osp-2, which instead has a non-polar residue (alanine[6]). The other two ostricacins, Osp-3 has 28 identical residues with Gal-7, whilst Osp-4 has 18 identical residues with Gal-8 (46.2% similarity). Osp-3 and Gal-7 characteristic motif at the amino terminus is comprised of one isoleucine, two prolines, one arginine, one aspartic acid and two varying residues. The varying residues include a large non-polar residue (leucine and isoleucine) and a small polar residue (proline and threonine). Osp-4 and Gal-8 show a characteristic motif of asn-glu-ala-gln-cys at the amino terminus. In contrast to Osp-1, all three ostricacins are not missing two residues between cysteine[8] and cysteine[15].

3.2.5 Antimicrobial activity of OSP-1-4

The minimum inhibitory concentrations (MICs) of these peptides were determined using the radial diffusion assay method (Table 3.4). This method is described in Chapter 2, Section 2.2.8. Amongst the four purified peptides, Osp-4 showed the weakest activity with the highest MIC values against the *E. coli* and *S. aureus*. Osp-1-3 appeared to be effective in killing both bacteria, as indicated by the low MIC values. In addition to the

activity against bacteria, Osp-2 also displayed activity against *C. albicans* with MIC value of 6.20 μg/ml.

Table 3.4 Minimum Inhibitory Concentrations (μg/ml) of Ostricacins-1-4.

Peptide	*E. coli* O157:H7	*S. aureus* 1056 MRSA	*C. albicans* 3153A
Osp-1	1.29	1.17	-
Osp-2	0.96	1.25	6.20
Osp-3	2.41	2.78	-
Osp-4	12.03	11.48	-

The antimicrobial activity test indicates that the ostricacins are effective to inhibit growth of both Gram-negative bacteria and Gram-positive bacteria, but not so effective against the yeast. The MIC values of Osp-1-4 against the two bacteria are as active as the other avian β-defensins, with MIC ranging from 0.5-16 μg/ml (Evans *et al.*, 1995),(Thouzeau *et al.*, 2003). Ostricacins-1-3 also appear to be as potent as the chicken gallinacins (Gal-1, 1α & 2), which are efficient in inhibiting growth of *E. coli* and *L. monocytogenes* (Harwig et al., 1994). These gallinacins have shown activity as potent as the rabbit neutrophil peptide NP-1 and stronger activity than the human HNP-1. These ostricacins show more potency than the thirteen bovine neutrophil β-defensins (Selsted *et al.*, 1993). However, amongst the avian β-defensins, only Osp-2, CHP-1 and THP-1 have shown potent activity against *C. albicans*. Investigation into the bactericidal effects of the avian β-defensins has only been done with sphenisin-2, which showed bactericidal activity mainly against Gram-positive bacteria (Thouzeau *et al.*, 2003). Sphenisin-2 was also the only avian β-defensins that demonstrated activity against filamentous fungi.

3.2.6 Amino Acid Composition Analysis

Compositions of these ostricacins were analysed using ExPASy ProtParam tools (http://us.expasy.org/tools/protparam.html), a website based programme used for examining protein physical and chemical parameters (Table 3.5). The four ostricacins

were rich in glycines, cysteines, and basic amino acids (arginines and lysines), as indicated in bold (Table 3.5).

Table 3.5 Amino Acid Composition of Ostricacins-1-4 (mole percent).

Amino acid	Osp-1	Osp-2	Osp-3	Osp-4	Amino acid	Osp-1	Osp-2	Osp-3	Osp-4
Ala	5.6 (2)	4.9 (2)	2.5 (1)	2.4 (1)	Leu	5.6 (2)	2.4 (1)	2.5 (1)	9.5 (4)
Arg	**5.6 (2)**	**4.9 (2)**	**17.5 (7)**	**11.9 (5)**	**Lys**	**8.3 (3)**	**12.2 (5)**	**2.5 (1)**	–
Asn	–	4.9 (2)	5.0 (2)	7.1 (3)	Met	–	–	–	–
Asp	2.8 (1)	–	2.5 (1)	–	Phe	11.1 (4)	14.6 (6)	5.0 (2)	4.8 (2)
Cys	**16.7 (6)**	**14.6 (6)**	**15.0 (6)**	**14.3 (6)**	Pro	5.6 (2)	4.9 (2)	10.0 (4)	7.1 (3)
Gln	–	–	2.5 (1)	4.8 (2)	Ser	2.8 (1)	7.3 (3)	2.5 (1)	4.8 (2)
Glu	–	7.3 (3)	–	2.4 (1)	Thr	2.8 (1)	–	5.0 (2)	2.4 (1)
Gly	**13.9 (5)**	**9.8 (4)**	**10.0 (4)**	**9.5 (4)**	Trp	5.6 (2)	2.4 (1)	2.5 (1)	2.4 (1)
His	5.6 (2)	–	–	2.4 (1)	Tyr	–	2.4 (1)	5.0 (2)	2.4 (1)
Ile	–	2.4 (1)	10.0 (4)	2.4 (1)	Val	8.3 (3)	4.9 (2)	–	9.5 (4)

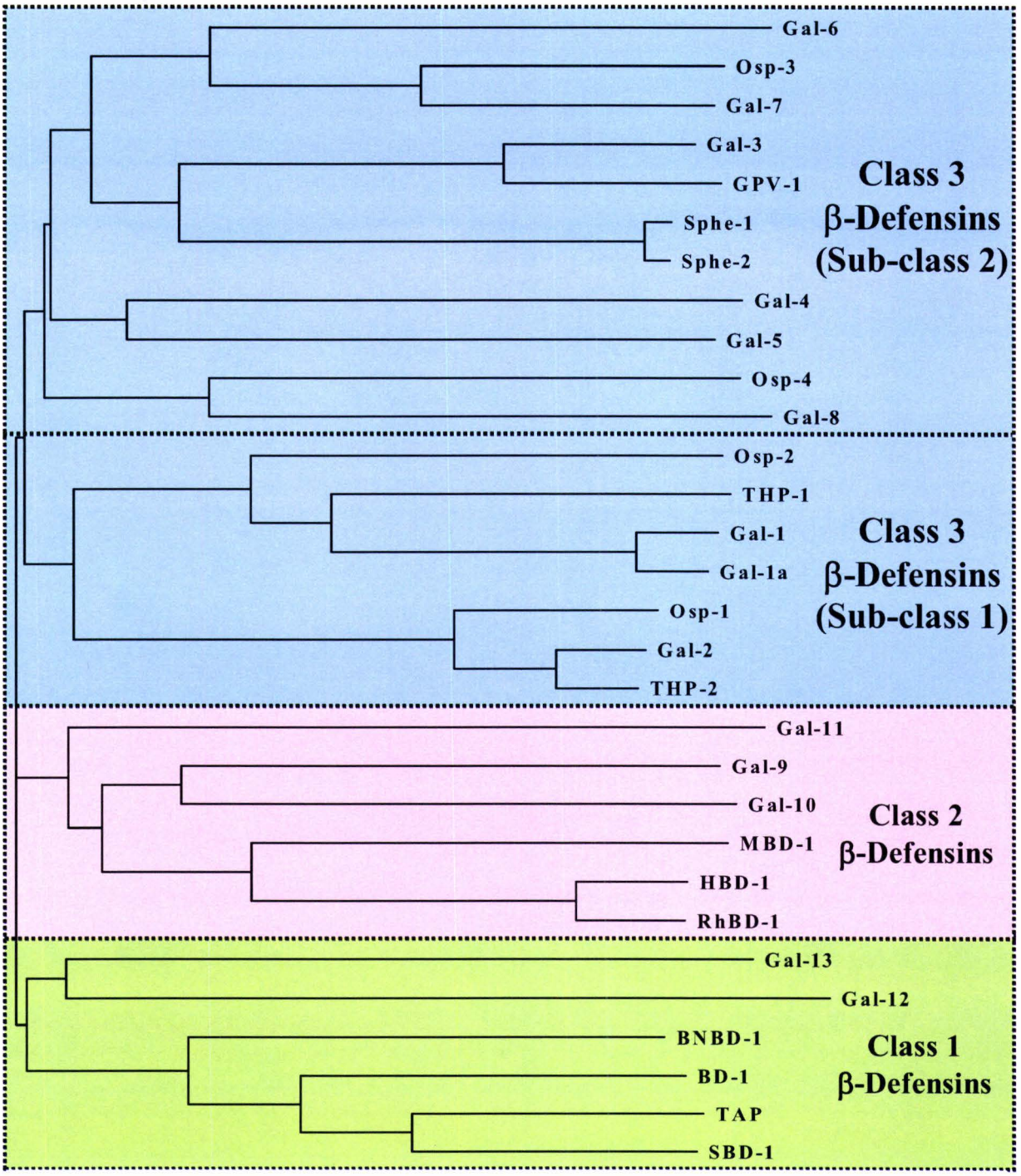
These four amino acids, glycines, cysteines, arginines and lysines, have also been commonly found comprising bovine β-defensins (Selsted *et al.*, 1993), chicken β-defensins (Evans *et al.*, 1994),(Harwig *et al.*, 1994), turkey β-defensins (Evans *et al.*, 1994), king penguin β-defensins (Thouzeau *et al.*, 2003), human β-defensins (Hoover *et al.*, 2001) and murine β-defensins (Klüver *et al.*, 2006). The significance of these residues in β-defensins is two-fold. Firstly, the cysteines and two glycines are essential structural residues, because they are components of the 'β-defensin core motif'. Secondly, the presence of arginine and lysine residues provides the peptides with a positive charge. With the addition of non-polar residues (alanines, phenylalanines, isoleucines, leucines and valines), β-defensins have an amphiphilic structure, which is the nature of host defence peptides that facilitates interactions with the negatively charged microbial surface structures, leading to inactivation of the pathogens.

Furthermore, Harwig *et al.* (1994) suggested that poor activity of Gal-2 against *C. albicans* was believed to be due to the truncation of three residues in the amino terminus and there being only four residues, instead of six, between the first and second cysteines that led to the loss of three basic amino acid residues (Harwig *et al.*, 1994). This idea can also be applied to the four ostricacins, where a correlation can be drawn between the ostricacins activity and the basic amino acid residues of the amino terminus. Osp-1, which is closely related to Gal-2, also has few residues truncated at the amino terminus and there is a loss of two residues between the first and second cysteine residues, whilst Osp-2, which is closely related to Gal-1 and Gal-1α, has longer residues in the amino terminus and retains six residues between the first and second cysteine residues. The missing residues, similar to the gallinacins, causes Osp-2 to have more positively charge residues at the C-terminal, hence it showed stronger activity against the three pathogens, particularly against *C. albicans*. For Osp-3 and Osp-4, the amino terminus do not lose any residues, but these two ostricacins have less basic residues than Osp-2. Osp-3 only has one arginine before the first cysteine, whereas Osp-4 does not have any basic residues before the first cysteine. These differences are believed to cause weaker antimicrobial activity.

3.2.7 Evolutionary Analysis of the Ostricacins

The FASTA database search, carried out previously, recognised many other β-defensins from mammalian and chicken. A phylogenetic analysis of the ostricacin was then carried out with Clustal X program, at which the ostricacins (Osp-1-4) were aligned with the chicken gallinacins (Gal-1-13), the turkey heterophil peptide (THP-1-2) and turkey epithelial β-defensin (GPV-1), the king penguin spheniscins (Sphe-1-2), bovine (BNBD-1 and TAP), sheep (SBD-1), pig (BD-1), murine (MBD-1), rhesus monkey (RhBD-1) and human β-defensin (HBD-1). A phylogenetic tree, which was based on the alignments of Clustal X, was generated to illustrate the evolutionary relationship between the four ostricacins and the avian and mammalian β-defensins (Figure 3.4).

Figure 3.4 Phylogenetic tree illustrating the homology of Osp-1-4 with other avian and mammalian β-defensins.



The phylogenetic tree has three variations, compared with Figure 1.13, which agreed with the classifications described in Zhang *et al.* (1998) (Zhang *et al.*, 1998). The first variation is the number of branches. Figure 1.13 shows two branches, of which the first branch deviates into two other branches, β-defensins class I and class II, whilst the

second branch, known as class III, is comprised of mostly avian β-defensins. Compared with Figure 1.13, Figure 3.4 instantly branches into the three classes.

The second variation is the presence of some avian β-defensins (gallinacins-9-13) in β-defensins class I and class II, which were not shown previously, because only gallinacins isolated from the chicken heterophils had been identified at the time when the classification was introduced. The heterophil gallinacins are not closely related to the mammalian β-defensins. On the other hand, Gal-9-11 are closely related to human, mouse and rhesus monkey β-defensins, whereas Gal-12-13 are closely related to cattle β-defensins, including the bovine, sheep and pig β-defensins. Therefore, these gallinacins can be classified into β-defensins class I and class II. Furthermore, the clustering of these gallinacins with the mammalian β-defensins supports the idea that these β-defensins have been around before the mammalian and avian lines diverged, which was estimated to be approximately 310 million years ago (Hedges *et al.*, 1996),(Kumar *et al.*, 1998),(Xiao *et al.*, 2004).

The last variation is in the subdivision of the third class, which is the avian β-defensins. In Figure 1.13, the division of the avian β-defensins was drawn between the β-defensins that had truncated amino acids in the amino terminuses (Osp-1, Gal-2, THP-2, etc) and the β-defensins that did not have truncated amino acids. In Figure 3.4, sub-class I is comprised of avian β-defensins isolated from the heterophils, whereas sub-class II is comprised mostly of avian β-defensins that are not isolated from heterophils, except for Osp-3 and Osp-4. Gal-4-7 have been reported to be expressed in bone marrow cells, whereas Gal-8 was reported to be expressed in epithelial cells (Xiao *et al.*, 2004).

Furthermore, the phylogenetic tree gives further insight into the evolutionary history of β-defensins. As discussed in the Literature Review, since the ostrich is considered to be the oldest order of flightless birds, the purifications of ostricacins suggests that β-defensins were phylogenetically an ancient effectors molecule of innate immunity, which would have around for at least 90 million years when the ratite species began to diverge (Cooper *et al.*, 1992),(van Tuinen *et al.*, 1998). This idea is strongly supported by the relatively even distribution of the four ostricacins through the avian β-defensins class. In addition, purification of the four ostricacins sustains the idea that avian host defence peptides are mainly composed of β-defensins. These avian β-defensins sequences are completely diverged, except for the "β-defensin core motif". This is

believed to be due to a positive Darwinian selection that causes these short molecules to vary significantly throughout the avian species (Xiao *et al.*, 2004),(Hughes and Yeager, 1997),(Hughes, 1999),(Ganz, 2005). The positive Darwinian selection caused even more variation, as the avian and mammalian species diverged. With different pathogenic microbial flora in each animal species, the diversification at the amino acid level was believed to have caused the emergence of α-defensins in mammalian species and θ-defensins in primates.

3.3 Conclusion

Four host defence peptides, namely ostricacins-1-4 were successfully purified from ostrich heterophils, using a combination of cationic exchange chromatography and RP-HPLC. As hypothesised, these ostricacins were β-defensin molecules characterised with the six-cysteine residues, which formed intramolecular disulfide bonds. The molecular weights of these ostricacins ranged from 4000-5000 Da. The ostricacins' amino acid sequences were highly diverged, with eight conserved residues, including the six-cysteines and two glycines. These conserved residues are known as the "β-defensin core motif".

The antimicrobial activity test, using the sensitive radial diffusion plate assay, showed that the ostricacins were effective inhibiting the growth of *E. coli* O157:H7 and *S. aureus* 1056MRSA, but they were not so effective against *C. albicans* 3153A. Only ostricacins-2 showed potency against the yeast. This activity against the yeast was related to the number of basic amino acids in the amino terminus, because ostricacins-2, along with gallinacins-1 and turkey heterophil peptide-1, retained more basic residues in the amino terminus than the other avian β-defensins. MIC values of these ostricacins, against the three pathogens, range from 0.96-12.03μg/ml. The antimicrobial activity was similar to the chicken gallinacins and the king penguin sphenisein-2. Compared with the MIC values of the mammalian β-defensins, they appeared to be as potent as the rabbit neutrophil peptide NP-1 and they were more potent than the human HNP-1 and the thirteen bovine neutrophil β-defensins (Selsted *et al.*, 1993),(Harwig *et al.*, 1994).

An analysis of the amino acid composition revealed that the ostricacins were rich in glycines, cysteines, arginines and lysines. Glycines and cysteines were believed to play a significant role in stabilising the β-defensins structure, whilst arginines and lysines were believed to provide the cationic charge of the β-defensins. These basic residues together with the hydrophobic residues are important in order to provide an amphiphilic structure, which plays a significant role in inactivating pathogens.

Evolutionary analysis, which was based on the alignment of ostricacins sequences with the other avian and mammalian β-defensins, yielded a phylogenetic tree, showing a division of vertebrate β-defensins into three classes. This classification was different in comparison to the classification introduced by Zhang *et al.* (1998). Zhang *et al.* (1998)

divided vertebrate β-defensins into avian β-defensins and mammalian β-defensins, in which the mammalian β-defensins were differentiated into two more classes (Zhang *et al.*, 1998). The phylogenetic tree divided vertebrate β-defensins into three classes. Furthermore, gallinacins-9-13 could be classified into the β-defensins class I and class II, based on the phylogenetic tree, because these avian β-defensins were closely clustered with the mammalian β-defensins. In addition, the four ostricacins were evenly distributed in the avian β-defensins class, which was differentiated into two subclasses. The first subclass was mainly comprised of avian heterophil β-defensins, whilst the second subclass, with the exception of ostricacins-3 and 4, was comprised of avian β-defensins expressed in the non-heterophil cells.

The clustering of avian and mammalian β-defensins indicated that β-defensins would have been around before the mammalian and avian lines diverged, approximately 310 million years ago. After the divergence, the β-defensin genes would have undergone positive Darwinian selection at the amino acid level, which caused the high variations of β-defensins sequences in the entire vertebrates. The positive Darwinian selection was also believed to be the cause of the emergence of α-defensins in mammalian species and θ-defensins in primates.

Chapter 4

Investigation of Antagonist Factors Affecting Antimicrobial Activity of Ostricacins-1 and 2 on *E. coli* O157:H7 and *S. aureus* 1056MRSA

4.1 Introduction

Most body fluids of vertebrates, such as sputum, milk, airway surface liquid, blood and serum/plasma, are known to contain 100-150 mM of sodium ions (Na^+/K^+) and 1-2 mM divalent cations (Mg^{2+}/Ca^{2+}). These cations are commonly found in animals and in the human body as electrolytes. The balance of electrolytes is essential for the normal function of organs and cells in animals and in the human body. However, the presence of these cations has demonstrated antagonist effect on the antimicrobial activity of defensins (Evans *et al.*, 1995),(Martin *et al.*, 1995),(Yang *et al.*, 2001),(Ganz, 2003),(Ganz, 2005). This is believed to be due to the competing nature of these cations for a specific binding site on the surface of microbial cells, such as lipopolysaccharides (LPS) of Gram-negative bacteria. The presence of these cationic ions is known to neutralise the positive charge of the host defence peptides, which is required for the initial interaction with the microbial membrane. Therefore, under physiological conditions, defensins are more likely to occur in the phagocytic vacuoles of phagocytes, where concentrations of cation and salt are minimal. A number of studies have also reported the antagonist effect of salt, divalent cations and sodium alginate on the activity of other host defence peptides (Friedrich *et al.*, 1999),(Tam *et al.*, 2002),(Anderson and Yu, 2005),(Bowdish *et al.*, 2005),(McPhee and Hancock, 2005).

As described in Chapter 3, four ostricacins have been successfully purified and characterised from ostrich heterophils. Two of these ostricacins, Osp-1 and Osp-2, have demonstrated antimicrobial activity and homology to other chicken and turkey heterophil β-defensins. Therefore, in this chapter, Osp-1 and 2 were chosen for further investigation into the antagonist effect of cationic ions on antimicrobial activity against *E. coli* O157:H7 and *S. aureus* 1056MRSA. These two particular bacteria were chosen

because the *E. coli* strain is a well-known food-pathogen, whilst the *S. aureus* strain is a resistant strain of Gram-positive bacteria that causes infections ranging from skins abscesses to life-threatening conditions, such as endocarditis and toxic shock. The yeast *Candida albicans* 3153A, which was used in the preliminary study (Chapter 3), was not included in this part of study, because the ostricacins showed little or no antimicrobial effect against the pathogens. In addition to the cations, varying temperatures was also included in the study, because it is known that increased temperature can cause denaturation of protein and peptides. Therefore, the objective of was this part of research was to investigate the effect of monovalent cations, divalent cations and temperature on the antimicrobial activity of ostricacins-1 and 2 against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

4.2 Results and Discussion

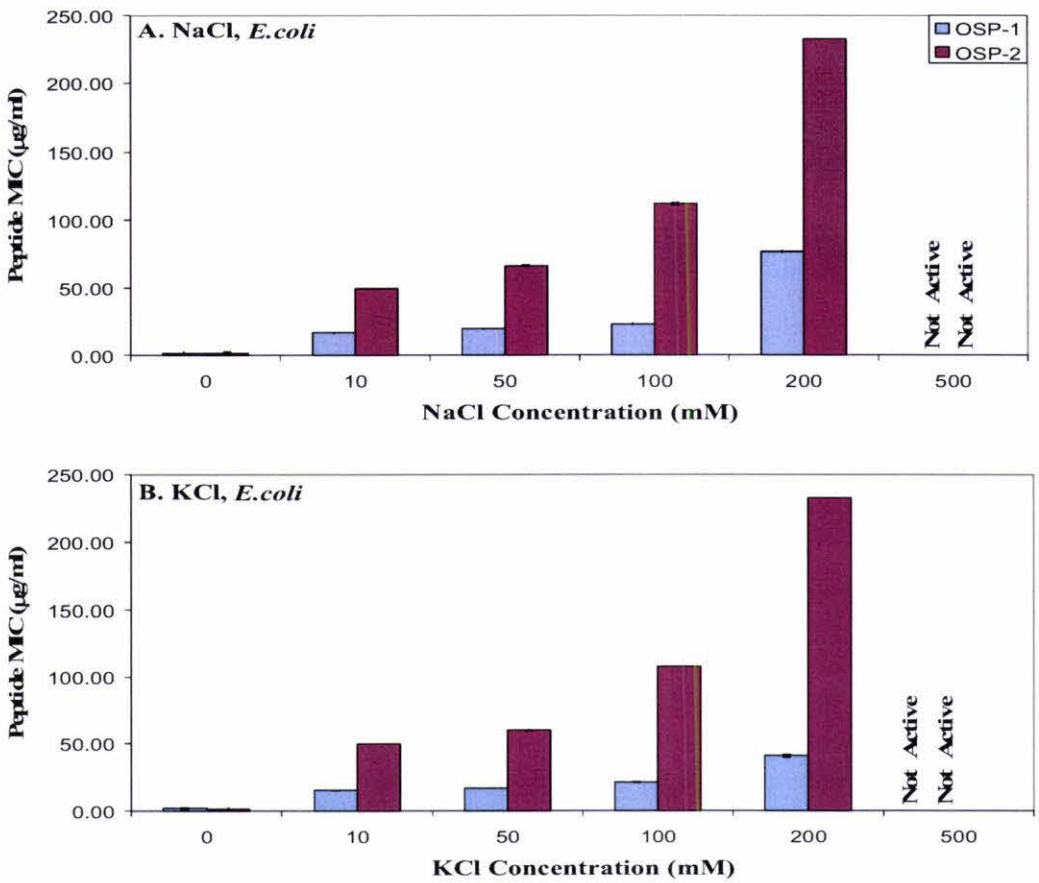
4.2.1 Effect of Monovalent Cations on E. coli O157:H7

The first antagonist factor investigated was monovalent cations (Na^+ and K^+). This investigation was carried out by determining the MIC values, using a modified radial diffusion plate assay method, as described in Chapter 2, Section 2.3.3. The monovalent cation concentrations varied between 0 mM and 500 mM. These cations were prepared as chloride salt solutions and they were then added into the melted underlay agar. The ostricacins were applied to the wells made in the underlay agar.

The results of the monovalent cation effect on the MIC of Osp-1 and Osp-2 on *E. coli* O157:H7 are shown in Figure 4.1, which displays the MIC of both ostricacins as geometric means of the three runs. An example of the MIC calculation can be found in Appendix 1, Section 1.1. Figure 4.1 also displays error bars, indicating a 95% confidence interval of each MIC value. However, the values of these error bars are too small to be seen (Appendix 1, Section 1.2). The significant effect of the monovalent cations on the ostricacins is indicated by a two-way analysis of variance (ANOVA) of the MIC, which was carried out with a 95% confidence interval on two parameters: the number of runs and the monovalent cation concentrations. The ANOVA results, shown in the Appendix 1, Section 1.3, suggest that the number of runs do not have a significant effect on the ostricacins' MIC values (p-value > 0.1), but the concentrations of monovalent cation have a significant effect on the MIC values (p-value < 0.05).

The average MIC values of Osp-1 and Osp-2 were 1.79 μg/ml and 1.49 μg/ml, respectively, when there were no cationic ions present (Figure 4.1). These values were referred to as the original MIC values. Furthermore, the monovalent cations affected the ostricacins' antimicrobial activity on the Gram-negative bacterium, which were indicated by the increasing MIC values. The MIC values of Osp-1 gradually increased (8-12-fold), as the concentration of Na^+/K^+ increased from 0 mM to 100 mM. A noticeable increase of MIC could also be seen at 200 mM Na^+ (42-fold) and at 200 mM K^+ (23-fold). The MIC values of Osp-2 increased more rapidly than the Osp-1 MIC (30-70-fold), as the concentration of Na^+/K^+ increased from 0 mM to 200 mM. At 200 mM, the Osp-2 MIC increased 150-fold from the original value. Both ostricacins lost their activity at 500 mM of the monovalent cations.

Figure 4.1 The effect of monovalent cations, Na^+ (A) and K^+(B), on the MIC of Osp-1 and 2 against *E. coli* O157:H7.



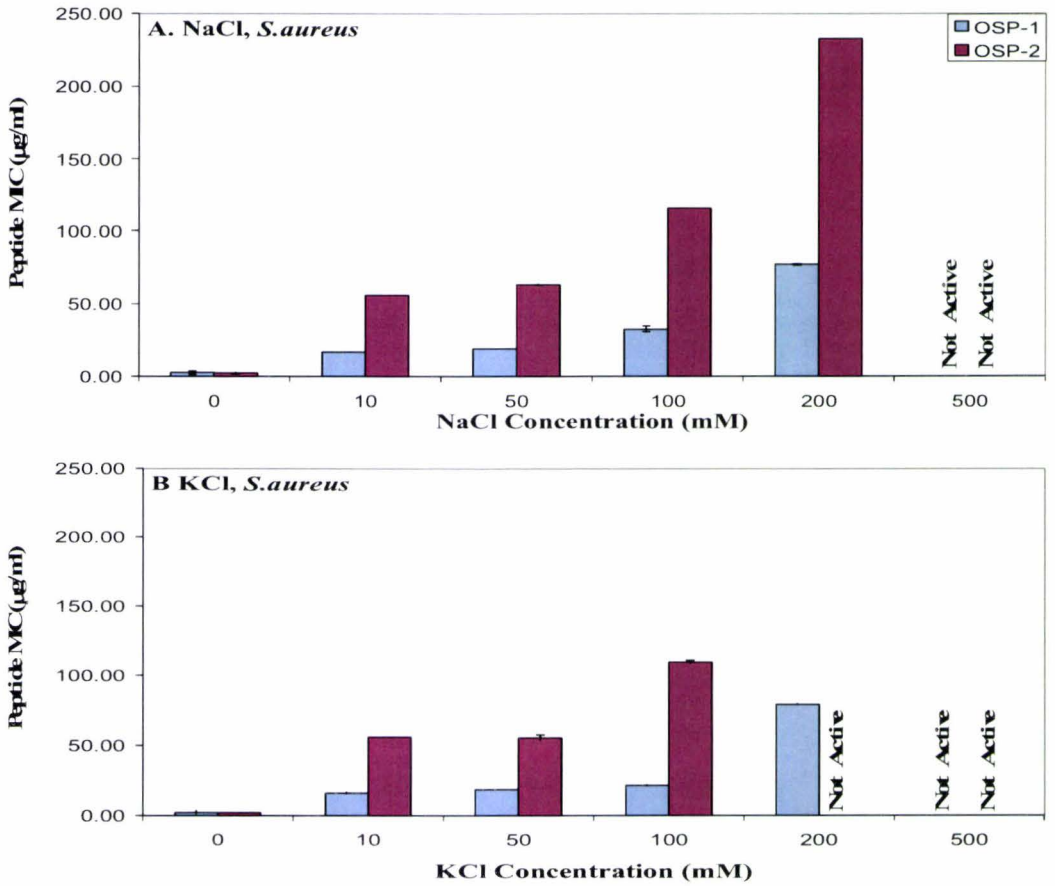
4.2.2 Effect of Monovalent Cations on S. aureus 1056MRSA

The results of the monovalent cation effect on ostricacins-1 and 2 MIC against *S. aureus* 1056MRSA are summarised in Figure 4.2, with error bars indicating 95% confidence intervals of the mean MIC values. However, the values of these error bars are too small to be seen (Appendix 1, Section 1.2). The ANOVA of the MIC (with 95% confidence interval) was carried out using the same two parameters described previously. These results suggest that the number of runs do not have a significant effect on the ostricacins' MIC values (p-value > 0.3), but the concentrations of monovalent cation have a significant effect on the MIC values (p-value < 0.05).

The original MIC values of Osp-1 and Osp-2 on *S. aureus* 1056MRSA were 2.48 μg/ml and 2.25 μg/ml, respectively (Figure 4.2). The monovalent cations also affected the ostricacins' antimicrobial activity on the Gram-positive bacterium. Osp-1 MIC values rose gradually, as the concentration of Na^+/K^+ increased from 0 mM to 200 mM. At 200

mM Na^+/K^+, the Osp-1 MICs increased 30-fold and it lost its activity at 500 mM. Furthermore, Osp-2 potency decreased more rapidly, with a 50-fold increase of MIC at 200 mM Na^+/K^+ and loss of activity at 500 mM. These results also showed that the activity of Osp-2 on *S. aureus* was markedly affected by potassium ions, because Osp-2 lost its activity at 200 mM K^+, whilst the peptide still retained moderate antimicrobial activity at the same concentration of Na^+.

Figure 4.2 The effect of monovalent cations, Na^+ (A) and K^+ (B), on the MIC of Osp-1 and 2 against *S. aureus* 1056MRSA.



The effect of monovalent cations on other avian β-defensin have not been investigated, apart from the king penguin sphenisin-2 (Sphe-2) (Landon *et al.*, 2004). Sphe-2 demonstrated potent antimicrobial activity against *E. coli* and *S. aureus* at 160 mM NaCl, but its potency against *S. aureus* decreased 16-fold at concentration 480 mM NaCl, whilst its potency against *E. coli* at 480 mM could not be determined, due to poor growth of the bacterium. A similar result was also shown by human β-defensin-3

(HBD-3) (Harder *et al.*, 2001). HBD-3 demonstrated a decrease of activity against *S. aureus* at 250 mM NaCl concentration, whilst it was still potent at physiologic salt concentrations (100-150 mM). The ability for Sphe-2 and HBD-3 to retain their potency at physiologic salt and high salt concentrations was believed to be due to the net positive charges, since Sphe-2 and HBD-3 have a net positive charge of +11 and +10, respectively. In comparison to Sphe-2 and HBD-3, the ostricacins potency, with a net positive charge of +4, declined at low NaCl concentration (10 mM) and the ostricacins demonstrated no activity at 500 mM.

A correlation between the degree of positive charge and the effect of monovalent cations on peptides' potency is maintained in other vertebrate β-defensins. Firstly, investigations of sodium chloride salts effect on human β-defensins-1 and human β-defensins-2 (HBD-1 and 2) demonstrated that the potency of HBD-1 and HBD-2 against *P. aeruginosa* and *E. coli* decreased, respectively, by increasing the concentration of sodium chloride (Goldman *et al.*, 1997), (Bals *et al.*, 1998b). There were also other animals β-defensins, such as canine β-defensins (cBDs) and mouse β-defensin-1 (MBD-1), which demonstrated decreasing activity against *E. coli*, with increasing sodium chloride concentrations (Bals *et al.*, 1998a),(Sang *et al.*, 2005). A similar result was also demonstrated by human HNP-1, an α-defensin whose potency was affected by 100 mM sodium ions (Turner *et al.*, 1998). These defensins, HBD-1 and 2, cBDs, MBD-1 and HNP-1 have lower net positive charges (+3, +4 or +6), when compared with Sphe-2 and HBD-3.

In addition, other host defence peptides, such as cecropin-bee melittin hybrid peptide (CEME) (Friedrich *et al.*, 1999), human LL-37 and porcine PG-1 (Turner *et al.*, 1998), and ovine SMAP-29, OaBac5mini and Oabac7.5mini (Anderson and Yu, 2005), have also demonstrated the effect of monovalent cations on the antimicrobial activity against various pathogens. Firstly, the activity of CEME variants, with positive charge between +5 and +7, demonstrated that their activities were not affected by the increasing sodium ions. Secondly, human LL-37 and porcine PG-1 demonstrated potency against all bacteria under low and high-salt conditions (100 mM NaCl). Thirdly, in the study of ovine peptides, only SMAP-29 potency was not affected by the increasing monovalent cation concentrations, whilst the other two OaBacs' antimicrobial activity decreased. In contrast to the β-defensins, these cathelicidins' antimicrobial activities were suggested

to be dependent on the flexibility of the peptides, as well as their hydrophobicity (Friedrich *et al.*, 1999).

These results indicate that the loss of the avian β-defensins' potency is believed to be due to interaction of the monovalent cations with the negatively charged bacterial surfaces, leading to the limitation of the peptides' binding ability to the bacterial surfaces. This binding ability is known to rely on the net positive charges (Martin *et al.*, 1995). Therefore, these β-defensins, with high net positive charges (≥+10), appear to be less affected by the increase of monovalent cations, compared with β-defensins with low net positive charges (<10).

4.2.3 Effect of Divalent Cations on E. coli O157:H7

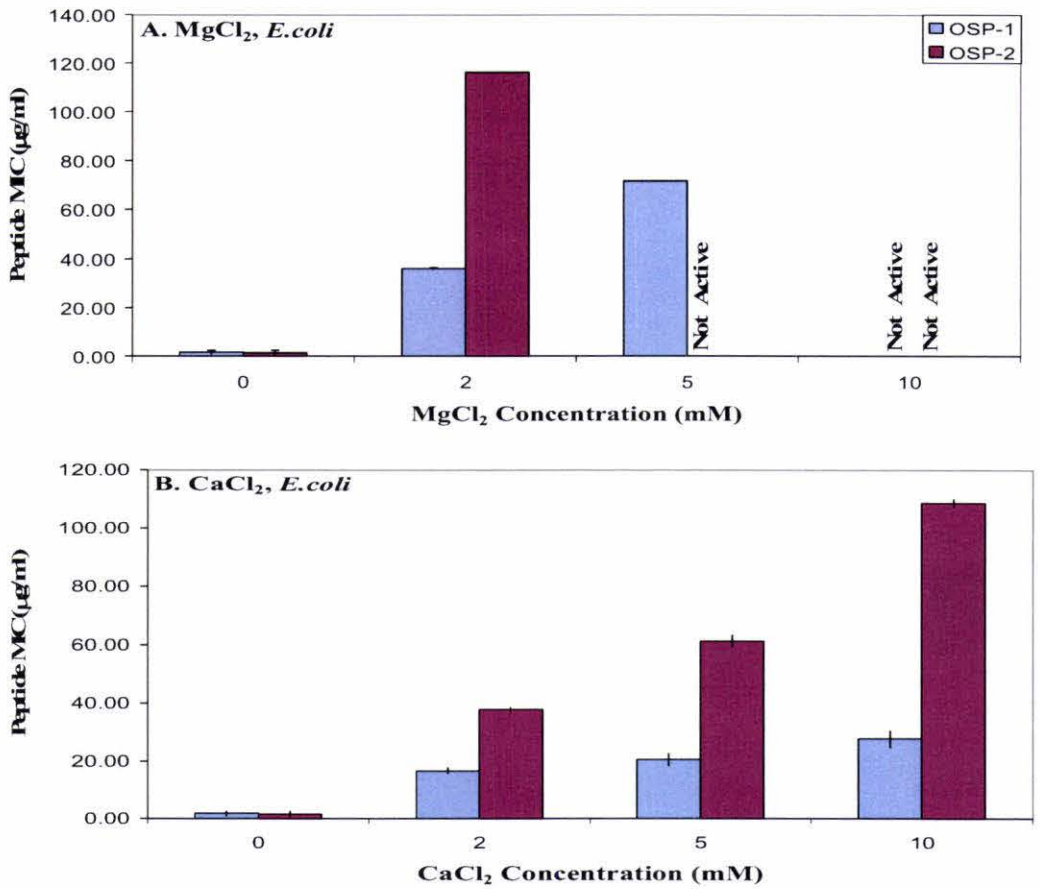
The second antagonist factor investigated was divalent cations (Ca^{2+} and Mg^{2+}). This investigation was carried out by determining the MIC values, using the modified radial diffusion plate assay method, as described in Chapter 2, Section 2.3.4. The divalent cation concentrations varied between 0 mM and 10 mM. These cationic ions were prepared as chloride salt solutions and they were then added into the melted underlay agar. The ostricacins were applied to the wells made in the underlay agar.

The results of the divalent cation effect on ostricacins-1 and 2 MIC against *E. coli* O157:H7 are shown in Figure 4.3, which displays the geometric mean of ostricacins-1 and 2 MIC. The error bars are also included, which indicate the 95% confidence intervals of the mean MIC values. However, the values of these error bars are too small to be seen (Appendix 1, Section 1.2). The ANOVA of the MIC values was also carried out using the same parameters. The results, presented in the Appendix 1, Section 1.3, are similar to the previous ANOVA results, suggesting that the number of runs do not have a significant effect on the ostricacins' MIC values (p-value > 0.5), but the concentrations of divalent cations have a significant effect on the MIC values (p-value < 0.05).

The magnesium ions demonstrated the most significant effect on both ostricacins' antimicrobial activity, because the MIC values increased rapidly, with Osp-1 showing no activity at 10 mM and Osp-2 showing no activity at 5 mM, whilst in the presence of 10 mM monovalent cations, these ostricacins indicated moderate activity. The calcium

ions also significantly affected the potency of ostricacins, with Osp-1 showing a 15-fold increase and Osp-2 showing a 72-fold increase of MIC between 0 mM and 10 mM Ca^{2+}.

Figure 4.3 The effect of divalent cations, Mg^{2+} (A) and Ca^{2+} (B), on the MIC of Osp-1 and 2 against *E. coli* O157:H7.

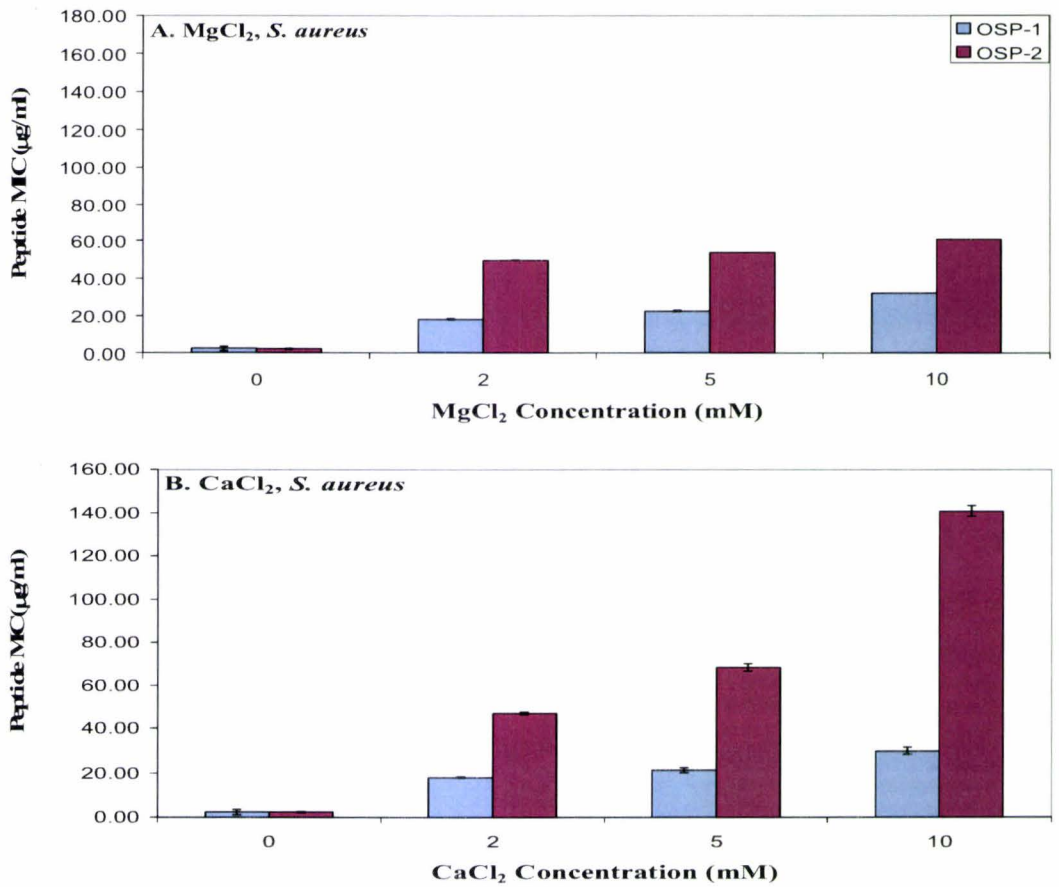


4.2.4 Effect of Divalent Cations on S. aureus 1056MRSA

The results of divalent cation effect on ostricacins-1 and 2 MIC against *S. aureus* 1056MRSA are summarised in Figure 4.4, which displays the geometric mean of both ostricacins MIC and error bars that indicate 95% confidence intervals. However, the values of these error bars are too small to be seen (Appendix 1, Section 1.2). The ANOVA results indicate that the number of runs does not cause a significant effect on the ostricacins' MIC values (p-value > 0.4), but increasing divalent cation concentrations have a significant effect on the MIC values (p-value < 0.05).

In addition, the divalent cations also affected the ostricacins' antimicrobial activity on the Gram-positive bacterium (Figure 4.4). However, the antagonist effect was not as strong as that shown by the effect of magnesium ions on the activity against *E. coli* O157:H7. The magnesium ions caused 7-fold and 20-fold increase on the MIC of Osp-1 and 2, respectively, at 2 mM. The MIC values indicated little change with increasing magnesium ion concentrations. A similar effect was also indicated by the calcium ions on Osp-1 antimicrobial activity. The calcium ions caused 7-fold increased on Osp-1 MIC at 2 mM. The MIC values remained relatively constant, with increasing calcium ion concentrations. On the contrary, Osp-2 MIC displayed a more rapid increase, with increasing calcium ion concentrations. At the highest concentration of calcium ion (10 mM), the MIC had approximately 60-fold increase.

Figure 4.4 The effect of divalent cations, Mg^{2+} (A) and Ca^{2+} (B), on the MIC of Osp-1 and 2 against *S. aureus* 1056MRSA.



In comparison with the monovalent cations, the divalent cations have a stronger effect on the ostricacins' antimicrobial activity. A similar trend in results have been reported with other host defence peptides, where the MIC values of the host defence peptides are more greatly affected by divalent cations than by monovalent cations (Turner *et al.*, 1998),(Friedrich *et al.*, 1999),(Anderson & Yu, 2005). The data relating to the divalent cation effect on other avian β-defensins have only been published in relation to the king penguin Sphe-2 (Landon *et al.*, 2004). This peptide was salt-resistant to increasing divalent cation concentrations. The potency of Sphe-2 against *S. aureus* was not affected at 1 mM Mg^{2+}, whilst a moderate decrease in the potency was observed at 50 mM Mg^{2+}. Furthermore, the potency of Sphe-2 against *E. coli* only decreased by two and four-fold, in the presence of 1 and 50 mM of Mg^{2+}, respectively. This avian β-defensin has shown ability to retain its activity in high divalent cation concentrations, due to the high overall net positive charge.

Furthermore, the ostricacins' potency against *E. coli* are strongly affected by divalent cations, as indicated by the loss of activity at 10 mM divalent cations, whilst the activity against *S. aureus* can still be observed at 10 mM divalent cations. These results agree with Martin *et al* (1995), who suggested that divalent cations substantially diminished defensins potency against Gram-negative bacteria, but these cations did not affect defensins potency against Gram-positive bacteria (Martin *et al.*, 1995). It is known that divalent cations have a high affinity to the LPS of Gram-negative bacteria. Therefore, the increasing concentrations of divalent cations with Gram-negative bacteria have more inhibitory effect on the host defence peptides. These results also indicate that the antimicrobial activity of ostricacins against Gram-negative bacteria is more sensitive in the presence of magnesium ions than calcium ions. In contrast to these ostrich β-defensins, cathelicidins have demonstrated more sensitivity in the presence of calcium ions than magnesium ions (Turner *et al.*, 1998),(Anderson & Yu, 2005). The reason that magnesium ions have a more significant effect on defensins may be due to the net positive charge and the secondary structure of defensins.

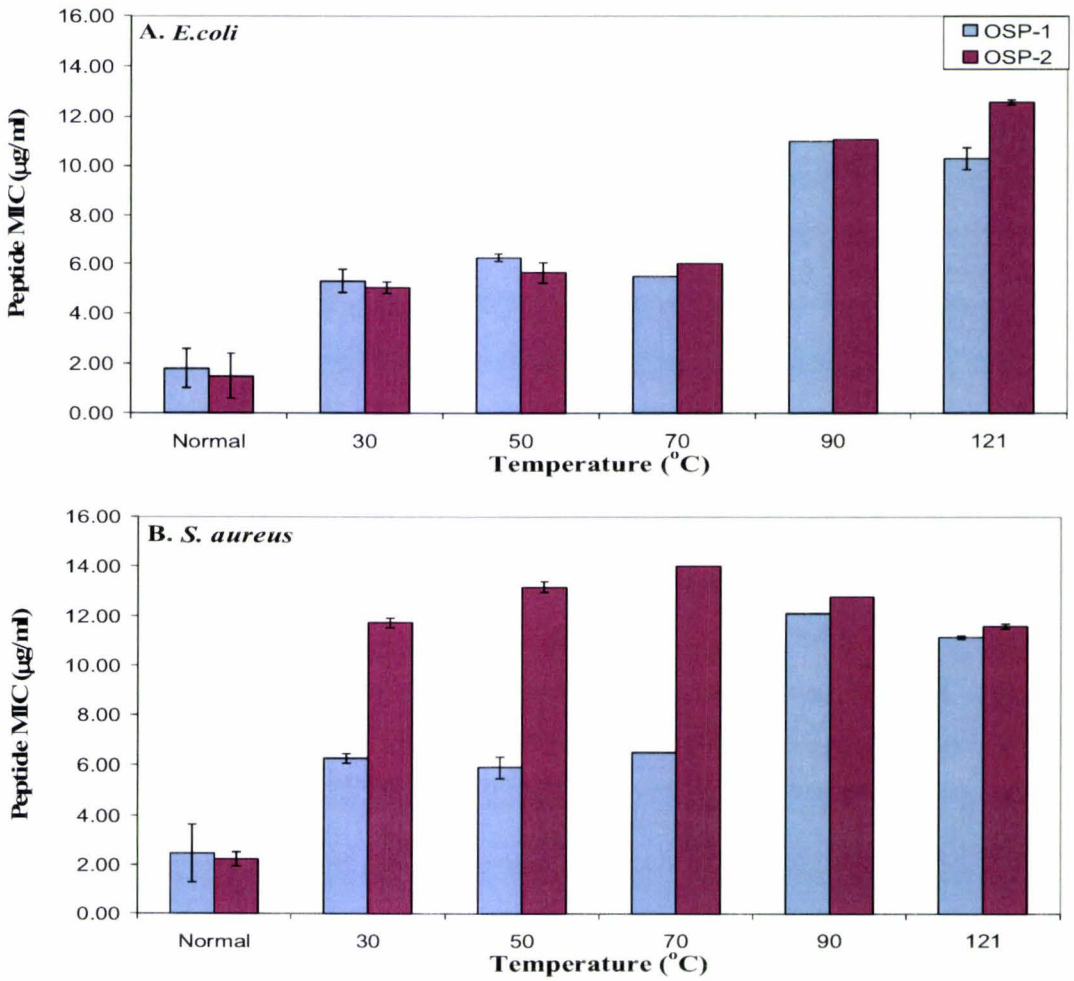
4.2.5 Effect of Temperature

The effect of temperature was also determined, using a modification of the radial diffusion plate assay method as described in Chapter 2, Section 2.3.5. The temperatures

included in this test were 30, 50, 70, 90 and 121°C (autoclave). The peptides were heated for 30 minutes at the desired temperatures and then they were applied to the underlay agar.

The results of temperature effect on ostricacins-1 and 2 MIC against both bacteria are shown in Figure 4.5, which displays the geometric mean MIC of both ostricacins and error bars that indicate 95% confidence intervals. However, the values of these error bars are too small to be seen (Appendix 1, Section 1.2). The ANOVA of these results indicate that the number of runs do not have a significant effect on the ostricacins' MIC values (p-value > 0.1), but the change in temperature had a significant effect on the MIC values (p-value < 0.05).

Figure 4.5 The effect of temperature increase on the MIC of Osp-1 and 2 against *E. coli* O157:H7 (A) and *S. aureus* 1056MRSA (B).



The results showed that the MIC of ostricacins against *E. coli* O157:H7 increased five-fold after being heated at 30°C (Figure 4.5). These values remained relatively stable, after being heated at 50°C and 70°C. However, after being heated at 90°C and after autoclaving, both ostricacins indicated a 10-fold increase of MIC. In addition, the antimicrobial activity of ostricacins against the *S. aureus* 1056MRSA demonstrated that Osp-2 is more affected by the temperature changes, compared with Osp-1. After being heated at 30°C, the increase of Osp-1 MIC was five-fold, whereas the MIC of OSP-2 had a 10-fold increase. The potency of Osp-2 remained relatively stable, even after autoclaving, whereas the potency of Osp-1 had a 10-fold decrease, after being heated at 90°C and after autoclaving.

In addition to this result, investigation of the antagonist effect of temperature has previously been shown using sheep host defence peptides (Anderson & Yu, 2005). Three ovine cathelicidins, OaBac5mini, OaBac7.5mini and SMAP-29, retained their potency against *E. coli* O157:H7 when heated at 80°C. These ovine peptides lost some activity after they were heated at 90°C for 30 minutes and after autoclaving, because the MIC values of OaBac5mini and SMAP-29 had an eight-fold increase, whilst the OaBac7.5mini had a four-fold increase. These results suggested that the thermal stability of host defence peptides was due to simple secondary structures.

Treatment involving heat is known to cause protein or peptide denaturations, which then leads to the loss of protein or peptide biological activity. Since ostricacins and cathelicidins were able to retain antimicrobial activity after heat treatment, host defence peptides appeared to be heat stable molecule. The stability was believed to be due to the secondary structure these molecules possess that could not be easily denatured. With ostricacins, the reduction of antimicrobial activity was believed to be due to the loss of disulfide bridges and other amino acid residues that contribute to the overall molecule heat stability. As both ostricacins composed of different amino acid residues, the heat treatment led to an increase of Osp-1 MIC at 30°C and at 90°C for both *E. coli* and *S. aureus*, whilst Osp-2 MIC behaved similarly when tested against *E. coli*, but increased once at 30°C when tested against *S. aureus*.

Furthermore, the difference of heat treatment effect on the ostricacins and the cathelicidins activity against *E. coli* was believed to be due to different secondary structure. The cathelicidins exist as a random structure in aqueous solution, whereas the ostricacins exist as β-sheet structure with the three stabilising disulphide bridges.

Therefore, the more robust structure of ostricacins was more sensitive to heat treatment than the more simple structure of cathelicidin.

Overall, these experiments indicated that amongst the two ostricacins, Osp-2 antimicrobial activity against both types of bacteria was more sensitive to changes in cationic concentrations and changes in temperature. Even though the antimicrobial activity of Osp-2 was slightly more potent than Osp-1, without the presence of cationic ions and at room temperature, the activity changed quite substantially with increasing cationic ion concentrations and with increasing temperatures. Therefore, it can be proposed that the sensitivity of Osp-2 activity may be related to the peptides' secondary structures. Confirmation of this hypothesis would require further investigation.

4.3 Conclusion

Ostricacin-1 and 2 were used in this part of the investigation. Both peptides were purified from the cation exchange and from the semi-prep RP-HPLC columns. A synthetic production of these ostricacins was attempted, but it appeared that the cost of synthesising β-defensins was too expensive. The reason for the high synthesis cost was believed to be due to the complexity in getting the proper disulphide linkages.

Both ostricacins, purified from the semi-prep RP-HPLC column, were then used for the investigations of the cation and temperature effect on the ostricacins' antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria. These experiments were carried out using the radial diffusion plate assay method, because the broth dilution method required high-concentration of peptides, which could not be produced from the semi-prep RP-HPLC column.

The potency of ostricacin-1 and 2 on Gram-negative bacteria and Gram-positive bacteria was significantly affected by the presence of monovalent and divalent cations. The divalent cations have more inhibitory effect than the monovalent cations on the ostricacins' antimicrobial activity, particularly against the Gram-negative bacterium. The activity was lost at high concentrations of monovalent cations (500 mM) and at low concentrations of divalent cations (10 mM). In contrast, the monovalent and divalent cations showed same significant effect when the ostricacins were tested against the Gram-positive bacterium. This was believed to be due to the presence of divalent cations on the bacterial surface, which interacted with the lipopolysaccharides (LPS) (Martin *et al.*, 1995). In comparison to other animal and human defensins, it was found that the degree of positive charge played an important role in the sensitivity of the peptides. Defensins with high overall net charge appeared to be cationic-resistant, compared with defensins with low overall net charge (Harder *et al.*, 2001),(Landon *et al.*, 2004).

These experiments also demonstrated that the ostricacins were able to retain their potency against Gram-negative bacteria, when heated to temperatures below 90°C. Therefore, ostricacins could be considered heat-stable molecules. The antimicrobial activity of both peptides showed a little change when the peptides were heated at 30°C, 50°C and 70°C for 30 minutes. There was a slight declined when the heating temperature was increased to 90°C or after autoclaving. The antimicrobial activity of

ostricacin-1 behaved similarly against the Gram-positive bacterium, but the antimicrobial activity of ostricacin-2 against the Gram-positive bacterium, was affected after being heated at 30°C. The same decline in activity was observed when ostricacin-2 was heated at 50, 70, 90°C and after autoclaving. These temperature effects on antimicrobial activity were believed to be due to the loss of disulfide bridges and other amino acid residues that contribute to the overall molecule heat stability.

Finally, it can be concluded that the activity of ostricacin-2 were more affected by the presence of cations and temperature increases, compared with ostricacin-1. When there were no cationic ions present, the activity of ostricacin-2 was slightly more potent than ostricacin-1. However, in the presence of cationic ions or increasing temperatures, the activity of ostricacin-2 was significantly affected. Further experimental work is required to investigate the reasons why ostricacin-2 was more affected by the cationic ions and temperatures than ostricacin-1.

Chapter 5

Investigation of Mechanisms of Action of Ostricacins against Gram-Negative Bacteria

5.1 Introduction

The inactivation of microorganisms by host defence peptides, such as defensins, is caused by either membrane disruptions or interactions with intracellular components (Epand and Vogel, 1999),(Shai, 1999),(Powers and Hancock, 2003),(Papo and Shai, 2003),(Hancock, 2004),(Reddy *et al.*, 2004),(Brogden, 2005),(McPhee and Hancock, 2005). The outer membrane of bacteria contains components, such as lipopolysaccharides (LPS) for Gram-negative bacteria and lipoteichoic acid (LTA) for Gram-positive bacteria, which are known as the docking sites for host defence peptides (Scott and Hancock, 2000). Within these two bacterial types, Gram-negative bacteria have been commonly used in the investigation of the mode of action of host defence peptides. The affinity to LPS is caused by electrostatic interactions between the positive charged peptides and the negative charged LPS. This is considered as the initial step of membrane disruption. After the attachment, the peptides disrupt the outer membrane allowing them to transverse into the periplasm, in order to interact with the cytoplasmic membrane. The peptides will then lyse the cytoplasmic membrane, which appears to be a lethal event. When the peptides do not cause cytoplasmic membrane disruptions, they can translocate into the bacterial cytoplasm and interact with bacterial DNA, RNA or other intracellular components. This interaction will inhibit DNA and RNA synthesis, together with enzymatic activity, leading to an inhibition of bacterial growth.

Membrane disruption mechanisms have been investigated and illustrated in some α-defensins, such as the human defensin HNP-1. HNP-1 demonstrated an ability to permeabilise the outer membrane and the inner membrane of *E. coli* ML-35, under conditions supporting bactericidal activity (Lehrer *et al.*, 1989). HNP-1 also demonstrated an ability to inhibit synthesis of the bacterial DNA, RNA and protein. The antimicrobial activity of HNP-1 against the *E. coli* strain was believed to occur simultaneously through outer membrane permeabilisation, inner membrane

permeabilisation, inhibition of macromolecular synthesis and the loss of colony forming potential. In addition, HNP-2 has been reported as forming transmembrane pores on large unilamellar vesicles (LUV), made from anionic lipid POPG (palmitoyloleoylphosphatidylglycerol) (Wimley *et al.*, 1994). The peptide dimers caused multimeric pores formation in the vesicles, through four chronological steps: binding of the peptide to the vesicles; induction of aggregation; peptides fusion into the vesicles; and permeabilisation. In another study, involving rabbit defensins (NP-1-5), it was demonstrated that the peptides bound to LUV made from pure POPG or mixtures of POPG with neutral phospholipid (Hristova *et al.*, 1996). However, the rabbit defensins, which are monomeric in aqueous solution, did not form stable pores in the membrane, thus indicating dimer formation may have been critical for the formation of multimeric pores.

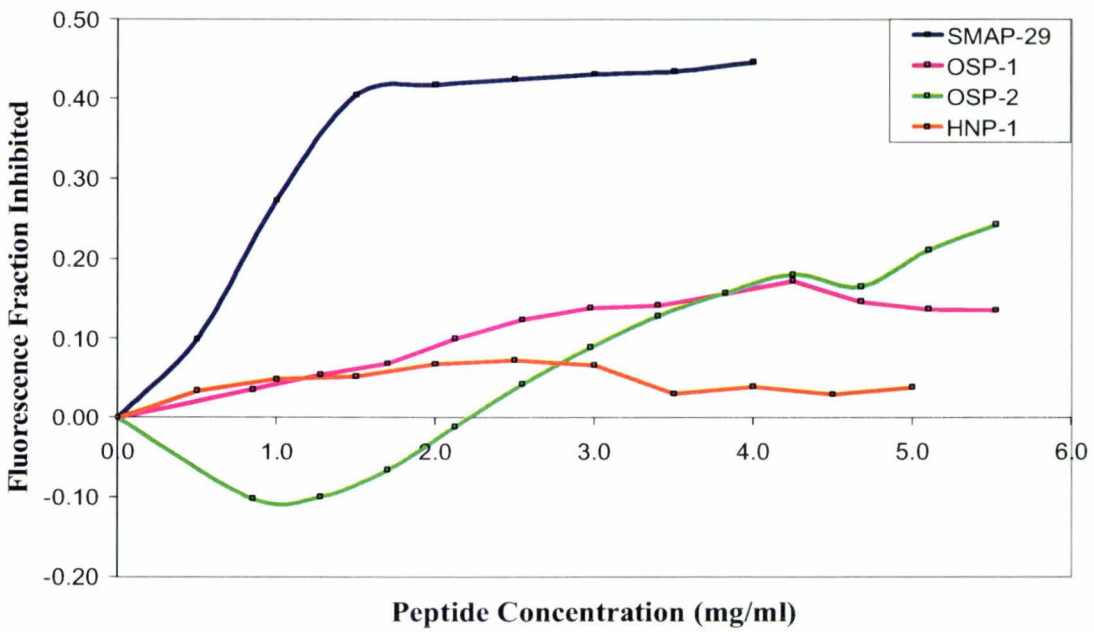
Four ostrich β-defensins have been isolated and named Osp-1-4. Two of these ostricacins (Osp-1 and 2) have appeared sensitive to the presence of cationic ions, whilst they have displayed stability to heat exposure under 90°C. In this part of the study, the objective was to determine the mode of action of the ostricacins on Gram-negative bacteria, based on three events: affinity to LPS; outer membrane permeabilisation; and cytoplasmic membrane depolarisation. A series of fluorescence assays were used to achieve this objective. These assays were developed by Dr Hancock's group at the University of British Columbia, Vancouver, Canada. The peptides were compared with a human neutrophil peptide (HNP-1) and a sheep myeloid antimicrobial peptide-29 (SMAP-29). SMAP-29 is a 29-residue peptide isolated from sheep, which has been well-characterised and used in previous researches (Anderson *et al.*, 2004),(Shin *et al.*, 2001),(Tack *et al.*, 2002). A kill curve was also included, in order to confirm whether the ostricacins caused cell lysis that led to loss of colony forming potential. In addition, a DNA gel electrophoresis was carried out, in order to investigate whether the ostricacins interacted with intracellular components.

5.2 Results and Discussion

5.2.1 LPS Binding Assay

For the purpose of testing the affinity of the ostricacins to LPS (the first step of membrane disruption mechanisms), a fluorescence assay was carried out using dansyl polymyxin B, a fluorescent dye that fluoresces when it is bound to LPS (Moore *et al.*, 1986),(Hancock *et al.*, 1991),(Anderson *et al.*, 2004),(Powers *et al.*, 2004). If the peptide binds to LPS, it will displace the dye, thus causing the fluorescence intensity to decrease. The assay was carried out in three replicates using Osp-1, Osp-2, HNP-1 and SMAP-29. This method is described in Chapter 2, Section 2.4.1. One of the results from these three runs is presented in Figure 5.1. The y-axis indicates the fluorescence fraction inhibited, which was calculated using Equation (2) (Chapter 2, Section 2.4.1), and the x-axis indicates an increasing concentration of the tested peptides. An example of the calculation of the fluorescence fraction inhibited is shown in Appendix 2, Section 2.1 and the results of the three assay runs are shown in Appendix 2, Section 2.2.

Figure 5.1 DPX/LPS binding assay of SMAP-29, HNP-1, OSP-1 and 2 indicating the affinity of the peptides to LPS.



All host defence peptides tested demonstrated a variation in degrees of affinity to LPS (Figure 5.1). The strongest affinity to LPS was displayed by SMAP-29, since it had the

highest increase of the fluorescence fraction inhibited with increasing peptide concentrations. This result has been previously reported (Anderson *et al.*, 2004). On the contrary, both ostricacins indicated a weak affinity to LPS, with the value of the fluorescence fraction inhibited being approximately half of the SMAP-29's, whereas HNP-1 displayed the weakest affinity amongst the tested peptides, with the smallest increase of fluorescence fraction inhibition.

The affinity of host defence peptides to the LPS appears to be related to the net positive charge of the host defence peptides. SMAP-29 contains ten basic amino acid residues, whereas Osp-1, Osp-2 and HNP-1 contain five, seven and four basic amino acid, respectively. The overall net positive charge for SMAP-29 is +9, whereas the net positive charge for the ostricacins and HNP-1 are only +4 and +3, respectively. As discussed in Chapter 4, low net positive charges caused defensins to lose potency in the presence of cationic ions. From this LPS binding assay result, it can be concluded that low net positive charges in host defence peptides lead to lower affinity to LPS, which significantly reduces the peptides' antimicrobial activity on Gram-negative bacteria in the presence of cationic ions. In addition, SMAP-29 has also been reported to contain two LPS binding sites that are located at each end of the peptide (Tack *et al.*, 2002). These two binding sites enable the peptide to bind cooperatively to LPS and they are believed to enhance the peptide's affinity to LPS. With low affinity to LPS, the ostricacins were expected to have a poor effect on the outer membrane of Gram-negative bacteria.

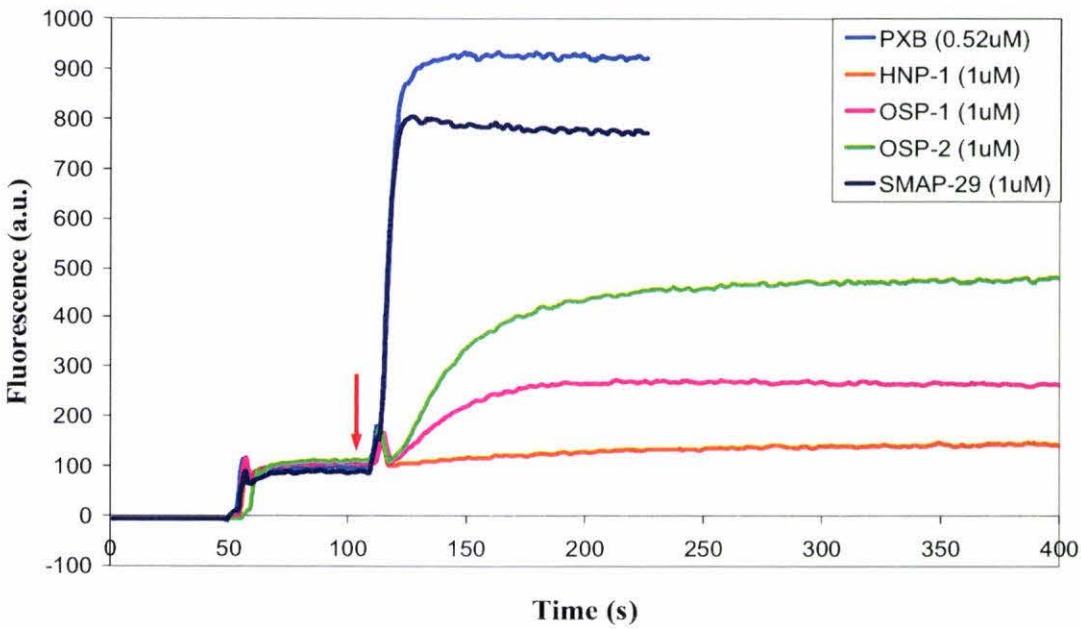
5.2.2 Outer Membrane Permeabilisation Assay

In order to test the ostricacins' ability to permeabilise the outer membrane, a second fluorescence assay was carried out, by measuring the uptake of a fluorescent dye, 1-*N*-phenylnapthylamine (NPN) (Loh *et al.*, 1984),(Hancock *et al.*, 1991),(Zhang *et al.*, 2000),(Yenugu *et al.*, 2003),(Anderson *et al.*, 2004),(Powers *et al.*, 2004). NPN is a small hydrophobic molecule dye that is normally excluded by the intact bacterial outer membrane. It will emit fluorescence, once it translocates itself into the periplasm, as the result of the disruption of outer membrane integrity by the host defence peptides. The strain *E. coli* UB1005 and the four peptides, Osp-1, Osp-2, HNP-1 and SMAP-29, were used in this assay, which was carried out in three replicates. A peptide antibiotic,

polymyxin B, was included, as the positive control. This method is described in Chapter 2, Section 2.4.2.

The results of the three runs are shown in Appendix 2, Section 2.3. One of these results is presented in Figure 5.2, which shows the kinetics of fluorescence intensity that change over time. This figure displays an initial uniform increase after 50 seconds, which is caused by the addition of NPN dye, and the second increase is caused by the addition of host defence peptides (red arrow). The second increase varies between host defence peptides, depending on the effect of each peptide on the bacterial outer membrane.

Figure 5.2 Kinetics of NPN uptake assay using *E. coli* UB 1005 that was treated with SMAP-29, HNP-1, OSP-1, OSP-2 and Polymyxin B (PXB). Red arrow indicates addition of peptides.



Three replicates demonstrated that SMAP-29, Osp-1 and Osp-2 caused NPN uptake, indicating that the three peptides caused disruption of the outer membrane of *E. coli* UB1005 (Figure 5.2). However, the degree of disruption varied between SMAP-29 and the two ostricacins. SMAP-29 had the maximum uptake of NPN, as indicated by the highest fluorescence intensity. The increase of fluorescence intensity was also rapid, implying that there was instantaneous outer membrane permeabilisation, which led to the rapid uptake of NPN dye into the periplasm. Furthermore, the results suggested that

the antimicrobial activity of SMAP-29 was equal to the antibiotic, because the fluorescence intensity of SMAP-29 appeared to be as strong as the positive control. Both assays of polymyxin B and SMAP-29 were stopped earlier than the defensins, because they had reached a stable plateau in a short period of time (approximately three to four minutes). SMAP-29 has been reported as causing a disruption of the outer membrane of *E. coli* UB1005 (Anderson *et al.*, 2004).

The ostricacins demonstrated a slower NPN uptake than SMAP-29 (approximately 2 minutes after the addition of the peptides), thus indicating that outer membrane permeabilisation also took place, but the effect of ostricacins on disrupting the outer membrane was not as instantaneous as that of SMAP-29. Both ostricacins also displayed a moderate increase of fluorescence intensity. This result suggested that ostricacins might only cause a partial disruption of the bacterial outer membrane. Furthermore, in all three runs, the fluorescence intensity of OSP-2 was always slightly higher than OSP-1, indicating a possibility that OSP-2 might have a more disruptive effect on the bacterial outer membrane than OSP-1, leading to more NPN uptake. Lastly, HNP-1 did not demonstrate any increase of fluorescence intensity, which suggested that it did not have any disruptive effect on the outer membrane of the Gram-negative bacterium.

The disruption of Gram-negative bacterial outer membrane is believed to be due to the affinity of the host defence peptides to LPS, as described in the membrane disruptive mechanism model. SMAP-29, which has the highest affinity to LPS, shows the most disruptive effect on the outer membrane of *E. coli* UB1005, whereas both ostricacins, which have a moderate affinity to LPS, display partial disruption of the outer membrane and HNP-1, that has the weakest affinity to LPS, causes no disruption of the outer membrane. The affinity of host defence peptides to LPS also seems to determine the time required by host defence peptides to cause outer membrane disruption. Host defence peptides with high affinity to LPS, such as SMAP-29, cause instantaneous disruption.

Osp-1 and 2 have shown low affinity to LPS and partial permeabilisation of the outer membrane of the Gram-negative bacteria. Therefore, prior to the next fluorescence assay, it was hypothesised that the ostricacins would cause little or no depolarisation of the cytoplasmic membrane. This second fluorescence assay has also presented a possibility that the ostricacins do not inactivate Gram-negative bacteria through

membrane disruption mechanisms; instead, their antimicrobial activities are due to interaction with intracellular components. Moreover, the inability of HNP-1 to permeabilise the Gram-negative bacteria outer membrane suggested that this peptide would not cause cytoplasmic membrane depolarisation. The following subsection will illustrate the antimicrobial activities of both ostricacins and HNP-1 on the cytoplasmic membrane.

5.2.3 Cytoplasmic Membrane Depolarisation Assay

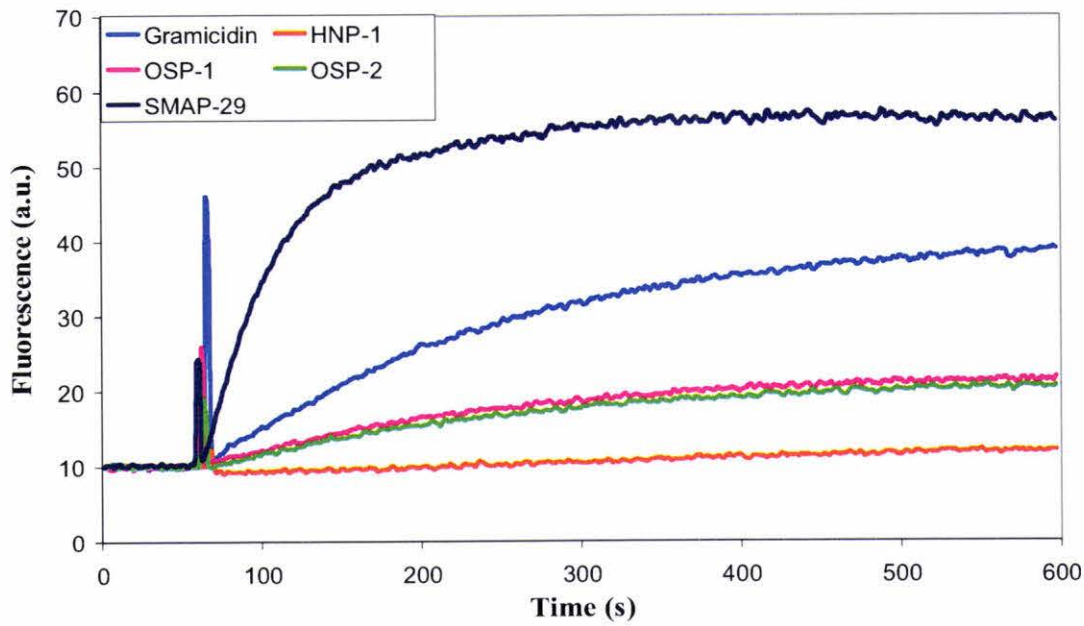
For the purpose of determining the cytoplasmic membrane depolarisation ability of host defence peptides, another fluorescence assay was carried out. This third fluorescence assay measured the fluorescence intensity caused by the release of a membrane potential sensitive dye, 3,3-dipropylthiacarbocyanine iodide ($diSC_35$), from the cytoplasmic membrane of *E. coli* DC2, an outer membrane hyperpermeable *E. coli* mutant (Wu and Hancock, 1999a),(Wu *et al.*, 1999b),(Zhang *et al.*, 2000),(Yenugu *et al.*, 2003),(Anderson *et al.*, 2004),(Powers *et al.*, 2004). This dye gets into the cytoplasmic membrane of *E. coli* DC2, where it becomes concentrated and self-quenches its own fluorescence. If the host defence peptides are able to form channels or disrupt the cytoplasmic membrane, the dye will be released and this causes an increase of fluorescence intensity.

The assay was carried out in three replicates, using the four peptides, Osp-1, Osp-2, HNP-1 and SMAP-29. Gramicidin is a peptide antibiotic, which was included as the positive control. This method is described in Chapter 2, Section 2.4.3. The results of these three replicates are shown in Appendix 2, Section 2.3. One of these results is presented in Figure 5.3, showing the kinetics of fluorescence intensity changes over time. It also shows that the addition of each host defence peptides is indicated with a peak, which has a fluorescence intensity of approximately 25-50 a.u.

The host defence peptides, except for HNP-1, have demonstrated that they depolarises the cytoplasmic membrane, thus indicating that they transversed the outer membrane of *E. coli* DC2 (Figure 5.3). The human α-defensins, as hypothesised, appeared not to be able to cause cytoplasmic membrane depolarisation, indicated by no increase of fluorescence intensity. SMAP-29 demonstrated the highest increase of fluorescence intensity, which suggested that substantial depolarisation of the membrane led to the

most displacement of $diSC_35$ dye. This peptide also displayed instantaneous depolarisation with the rapid fluorescence intensity increase. This result has been previously described, (Anderson *et al.*, 2004). SMAP-29 also displayed stronger fluorescence intensity than Gramicidin, suggesting that it might have worked better than the antibiotic. In addition, this result implied that Osp-1 and 2 caused low cytoplasmic membrane depolarisation, as indicated by the relatively low increase of fluorescence intensity. The effect of Osp-1 and 2 were also not as instantaneous as SMAP-29. These results of the ostricacins suggested that partial disruption of the cytoplasmic membrane took place. In contrast to the second fluorescence assay, both ostricacins appeared to have similar fluorescence intensity in the three assay runs.

Figure 5.3 Cytoplasmic membrane depolarisation that induced by SMAP-29, HNP-1, OSP-1, OSP-2 and Gramicidin.



The results regarding permeability of HNP-1 were different to that in the previous report, investigating the interaction of HNP-1 with *E. coli* ML35 (Lehrer *et al.*, 1989). Lehrer *et al* (1989) suggested that HNP-1 was able to permeabilise the bacterial outer and inner membrane, whereas, in our fluorescence assays, HNP-1 did not disrupt those membranes. These result differences are believed to be due to the different experimental methods being used. In the three fluorescent assays developed by Dr Hancock's group, the experiments were carried out in just a few minutes, whereas Lehrer *et al* (1989)

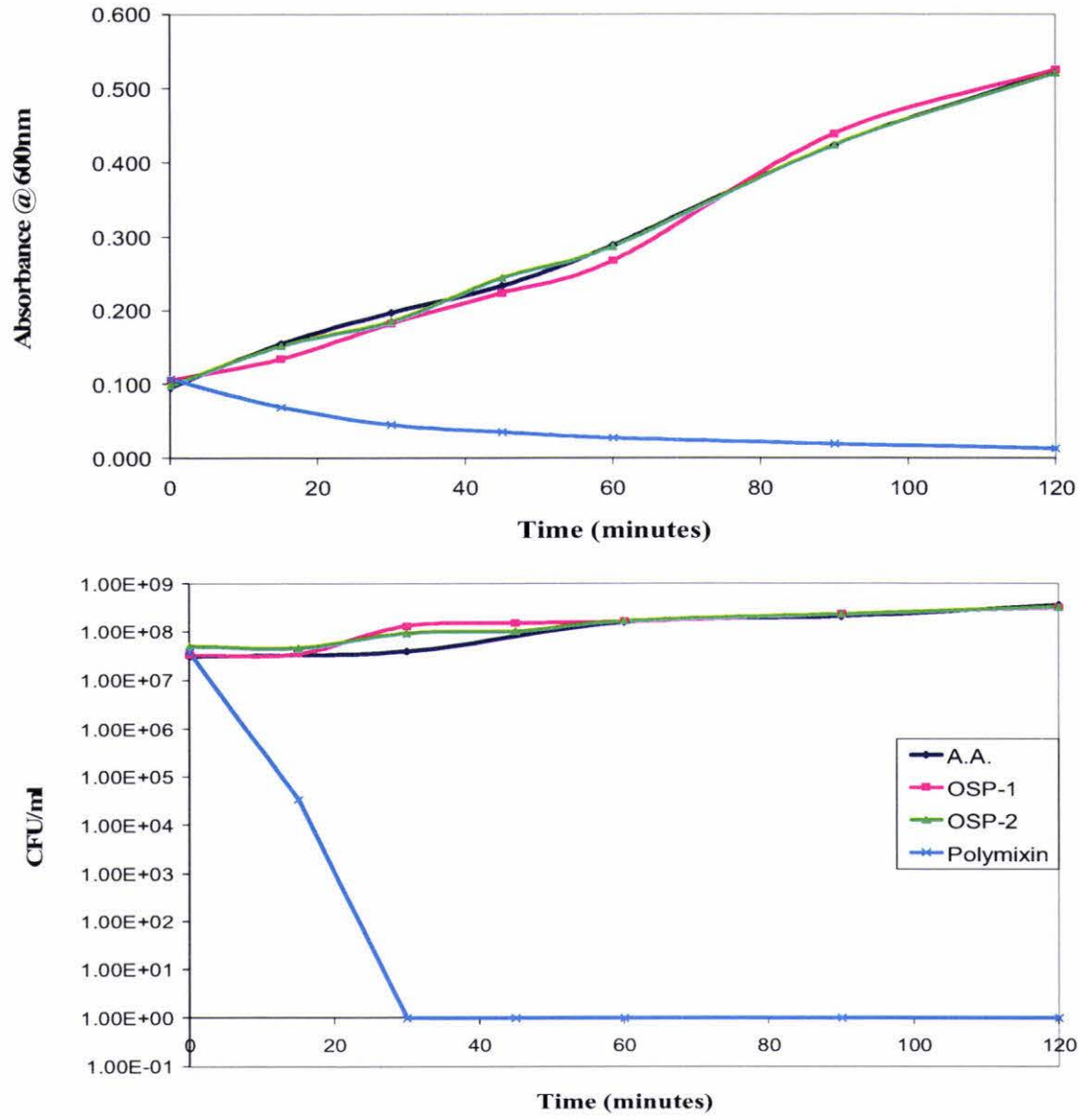
carried out the membrane permeabilisation assay for approximately 60-80 minutes. Therefore, if HNP-1 were meant to disrupt the bacterial outer and inner membrane, the disruption would not be seen in our fluorescence assays, because the assays were carried out in a shorter period of time. In addition, a β-defensin counterpart of HNP-1, human β-defensin-1 (HBD-1), demonstrated an ability to disrupt the outer membrane and the inner membrane within 1-hour and 2-hour, respectively, in an experiment that used the same fluorescence assays as this study (Yenugu *et al.*, 2003). Yenugu *et al* (2003) also used a HE2 peptide, which was able to rapidly disrupt both membranes (1-5 minutes). This result implied that HNP-1 and HBD-1 might have similar antimicrobial activity, at which time the disruption of the outer and inner membrane of Gram-negative bacteria did not occur instantaneously. Hence, when HNP-1 was investigated, in the same amount of time as SMAP-29, it was not able to disrupt both membranes of the Gram-negative bacteria.

In addition, these three fluorescence assays have indicated that the affinity of host defence peptides to LPS is the crucial step in membrane disruption mechanisms. Host defence peptides, which have a high affinity to LPS, such as SMAP-29, cause disruption of the Gram-negative bacteria outer membrane and depolarisation of the cytoplasmic membrane. SMAP-29 has also shown to be bactericidal, as they inactivated Gram-negative bacterial cells (Anderson *et al.*, 2004). So far, the ostricacins have shown the ability to inhibit bacterial growth with MIC 1-3 μg/ml (Chapter 3). Moreover, the fluorescence assays have displayed that the ostricacins have a weak affinity to LPS and they are able to cause moderate disruption of the Gram-negative outer membrane and depolarisation of the Gram-negative cytoplasmic membrane. These results suggested that the ostricacins cause disruption to both outer and cytoplasmic membrane. However, these results are not sufficient to determine whether the membrane disruptive mechanism causes the inactivation of bacterial cells. If the ostricacins are able to inactivate bacterial cells through membrane disruption, they will cause a decrease in viable cell numbers in a kill curve experiment. Otherwise, the disruption of cytoplasmic membrane only allows the ostricacins to translocate into the cytoplasm and interact with intracellular components. Therefore, a construction of a kill curve and a DNA gel electrophoresis were carried out.

5.2.4 Kill curve

The kill curve was divided into measurements of optical density and determination of colony forming unit (CFU). This method is described in Chapter 2, Section 2.4.4. Both tests were carried out in parallel, using *E. coli* O111, which was diluted to a concentration of approximately $2x10^7$ CFU/ml. Polymyxin B and 0.01% acetic acid were included as the positive and the negative control, respectively. These tests were also done in three replicates. The results of the three replicates are attached to the Appendix 2, Section 2.4. One of these results is presented in Figure 5.4.

Figure 5.4 Measurements of OD_{600nm} taken in parallel with the colonies counting of OSP-1 and 2 against *E. coli* O111.



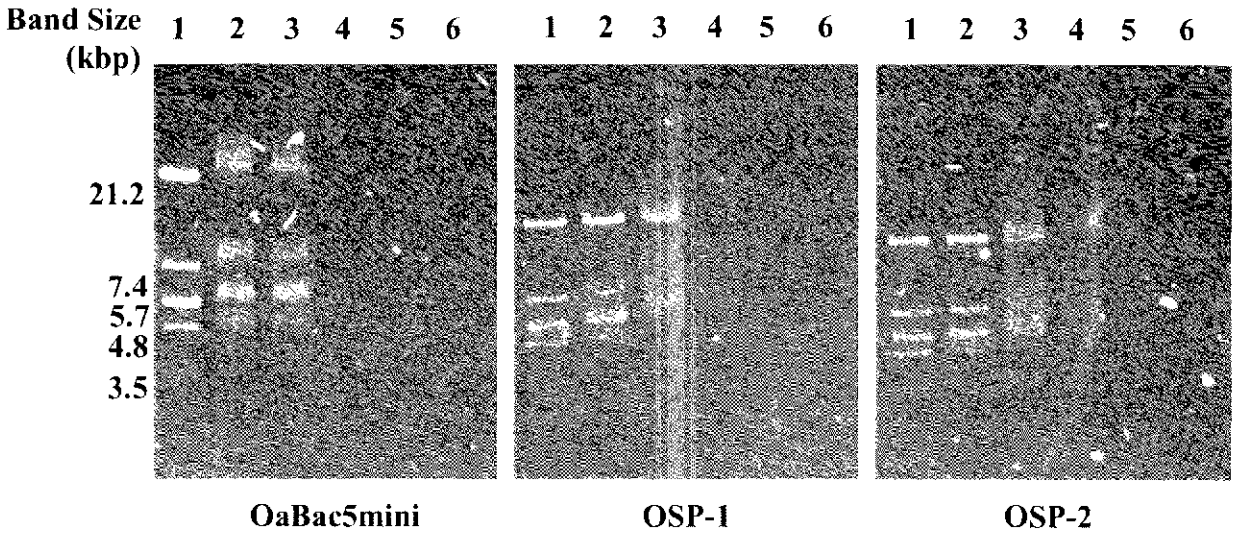
Both ostricacins demonstrated an inability to inhibit colonies formation and to limit the increase in optical density. They appeared to have the same trend as the negative control. The positive control was very effective in inhibiting the colonies formation potential of the Gram-negative bacterium, as it reduced the number of bacterial cell death by three-log in the first ten minutes and no viable cells remained after approximately thirty minutes. Since the ostricacins were not able to kill microbial cells in this experiment, the ostricacins' antimicrobial activity was not believed to be due to bacterial cell lysis, but instead, the activity was due to interaction with intracellular components of Gram-negative bacteria.

5.2.5 DNA Gel Electrophoresis

After both ostricacins were unable to induce bacterial cell death, a DNA gel electrophoresis was carried out to investigate whether interaction between the ostricacins and bacterial intracellular contents took place. The two ostricacins were compared with OaBac5 mini. The peptides were mixed with bacterial λDNA, which had been cut with the restriction enzyme EcoRI into several fragments sized 21.2 kbp, 7.4 kbp, 5.8 kbp, 5.6 kbp, 4.8 kbp and 3.5 kbp. This method is described in Chapter 2, Section 2.4.5 and the result is presented in Figure 5.5. The two similarly size bands, 5.8 kbp and 5.6 kbp, are shown as a 5.7 kbp band.

When the host defence peptide bound to the λDNA, it formed larger molecules that prevented their migration in the agarose gel (Figure 5.5). Both ostricacins appeared to bind to the bacterial λDNA, as they inhibited the λDNA migrations. The inhibition occurred at a minimum ratio of 1:2 between the λDNA and Osp-1 and at a minimum ratio of 1:4 between the λDNA and Osp-2. Both ostricacins were almost as effective as the ovine OaBac5mini that binds the λDNA at a ratio of 1:2. The binding ability displayed by these peptides suggested that they would most likely interact with the bacterial intracellular components

Figure 5.5 Interactions between different ratios of bacterial λDNA and the host defence peptide using DNA Gel Electrophoresis.
Lane 1, λDNA standard (DNA:Peptide ratio 1:0); Lane 2, DNA:Peptide ratio 1:0.5; Lane 3, DNA:Peptide ratio 1:1; Lane 4, DNA:Peptide ratio 1:2; Lane 5, DNA:Peptide ratio 1:4; and Lane 6, DNA:Peptide ratio 1:8.



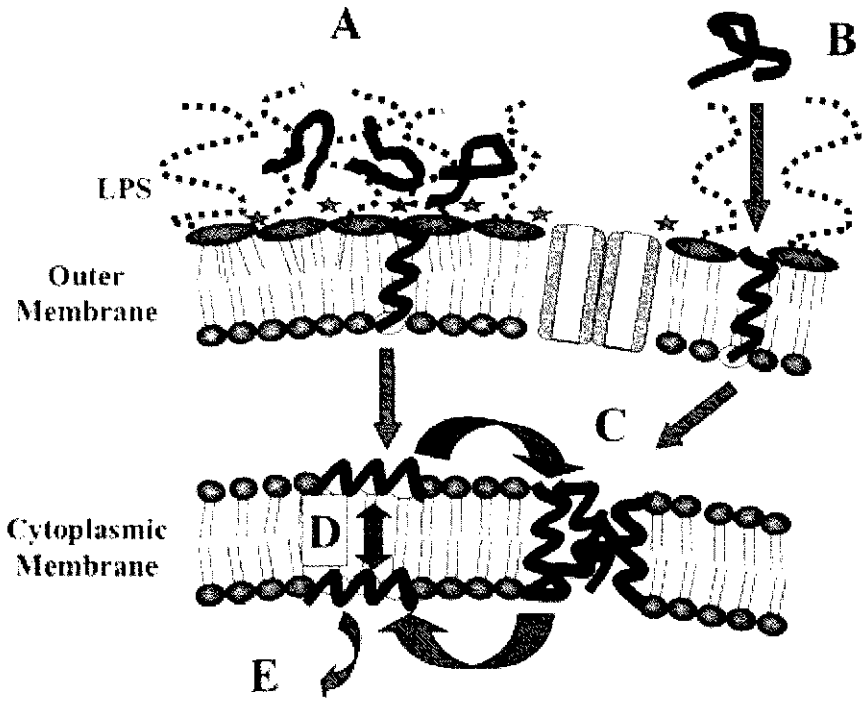
The two ostricacins have similar activity as OaBac5mini. OaBac5mini has been reported to inhibit growth of Gram-negative bacteria (Anderson and Yu, 2003), but its antimicrobial activity is affected by cationic ions (Anderson and Yu, 2005). This peptide has also displayed a low affinity for LPS and caused weak outer membrane permeabilisation and weak cytoplasmic membrane depolarisation (Anderson *et al.*, 2004). In comparison to OaBac5mini, Osp-1 and 2 have also displayed the ability to inhibit Gram-negative bacteria (Chapter 3). Furthermore, their antimicrobial activities are significantly affected by cationic ions (Chapter 4). As previously described, the ostricacins have a low affinity to LPS and they cause weak disruption of the outer and inner membrane of Gram-negative bacteria, which cannot cause lysis of the Gram-negative bacterial cells. In this experiment, OaBac5mini, as well as the ostricacins, were able to interact with the bacterial λDNA. Therefore, since OaBac5mini has been reported to function as bacteriostatic, these two ostricacins, which showed the same characteristics as OaBac5mini throughout a series of assays, are also bacteriostatic. The ostricacins are not able to directly kill Gram-negative bacteria through cell lysis, but they inhibit bacterial growth, due to the interaction of these peptides with intracellular components of the bacteria.

Papo and Shai (2003) suggested that the ability of antimicrobial peptides to cross the cell wall depended on their secondary and tertiary structures, as well as the oligomeric state (Papo and Shai, 2003). Therefore, besides affinity for LPS, the size and structural existence in solutions of host defence peptides would also affect the ability for host defence peptides to permeabilise the outer and inner membrane of Gram-negative bacteria. The smaller the molecule size, the easier it is to cross the barrier. Defensins molecules, such as HNP-1 and king penguin sphenisin-2, exist in solution as dimers and monomers, respectively (White *et al.*, 1995),(Landon *et al.*, 2004). These peptides have larger structures, compared to an α-helical cathelicidin, such as SMAP-29 that has less amino acid residues. Therefore, these defensins did not have the same ability to disrupt membrane integrity as the cathelicidin. Due to the size and structure in solutions, it can be hypothesised that defensins are most likely bacteriostatic peptides against Gram-negative bacteria, which exert their antimicrobial activity by interacting with intracellular components. Confirmation of this hypothesis would require further investigation.

From this part of the study, the bacteriostatic mechanism of the ostricacins against Gram-negative bacteria takes place through four chronological events: affinity to LPS; partial disruption of the outer and inner membrane; and interaction with intracellular components. These events, which are believed to occur simultaneously, depend on the strength of the peptides' affinity to LPS. A model illustrating this mechanism is shown in Figure 5.6. This model, described in the literature review, is developed by Dr Hancock's group and known as micellar-aggregate model (Hancock and Chapple, 1999),(Hancock, 2001),(Powers and Hancock, 2003). According to this model, the passing of host defence peptides into the cytoplasm is due to the disruption of the cytoplasmic membrane or they can 'flip-flop' across the cytoplasmic membrane, under the influence of the large transmembrane potential gradient. As shown in the results of this part of research, the ostricacins are initially attracted to the LPS due to the electrostatic interactions. It is believed that the affinity to LPS causes displacement of cationic ions and leads to partial disruption of the Gram-negative outer membrane. In the periplasm, the ostricacins cause partial disruption of the cytoplasmic membrane, which allow the peptide to get into the cytoplasm. The ostricacins then interact with the intracellular components, as shown by interaction of these peptides with bacterial λDNA. This interaction is believed to cause the inhibition of bacterial growth. An

observation, using transmission electron microscopy (TEM) and atomic force microscopy (AFM), will be useful to confirm these results.

Figure 5.6 Model illustrating the mechanism of ostricacins on Gram-negative bacteria (Hancock and Chapple, 1999),(Hancock, 2001),(Powers and Hancock, 2003).



In chronological order, (A) affinity of the ostricacins to LPS displacing the magnesium ions (shown as stars), followed by (B) the partial disruption of the outer membrane allowing the peptides to get into the periplasm. (C) The peptides then cause partial disruption of the cytoplasmic membrane or (D) 'flip-flop' across the membrane to get into the cytoplasm. In the cytoplasm, the peptides are able to interact with intracellular components to inhibit the biochemical reaction of the bacteria.

5.3 Conclusion

The three fluorescence assays indicated that the antimicrobial peptides membrane permeability depended on an affinity to LPS. When antimicrobial peptides had a strong affinity towards LPS, they were able to disrupt the outer membrane and cytoplasmic membrane. These events were demonstrated by the four peptides that have different affinities to LPS. SMAP-29, which showed the highest affinity, was able to rapidly permeabilise bacterial outer and cytoplasmic membrane. Osp-1 and 2, which had moderate affinity to LPS, caused partial disruption of the outer and inner membrane integrity, whilst HNP-1, which had the weakest affinity to LPS, did not show ability to permeabilise both membranes.

Furthermore, both ostricacins could not cause cell lysis that led to the killing of Gram-negative bacteria. The killing kinetics experiment suggested that the ostricacins did not inhibit the colony forming potential of Gram-negative. However, they appeared to be bacteriostatic peptides, because these peptides were able to inhibit bacterial growth, as shown in the previous Chapters. This ability was believed to be due to interaction of the ostricacins with the Gram-negative bacterial intracellular components. The ostricacins caused partial disruption of the outer and inner membrane of Gram-negative bacteria that allowed them to get into the cytoplasm. This mechanism is also shown by HNP-1.

In addition, the ability of HNP-1 to permeabilise the outer and inner membrane of Gram-negative bacteria was found to be very slow (approximately 1-2 hours). Therefore, in this experiment, HNP-1 was not able to demonstrate permeabilisation of both membranes, whilst in Lehrer *et al* (1985), it showed the ability to disrupt both membranes in a longer period of experimentation (Lehrer *et al.*, 1989).

Finally, it can be concluded there are two factors affecting the permeability of host defence peptides. The first factor is the overall net positive charge that affected the affinity to LPS. SMAP-29 has an overall net positive charge of +10 (Shin *et al.*, 2001), whilst the defensins, both ostricacins and HNP-1, only have an overall net positive charge of +4 and +3, respectively. Therefore, the SMAP-29 showed stronger affinity than the defensins. The second factor is the structure and size of host defence peptides, because the ability of host defence peptides to cross the cell wall depended on their secondary and tertiary structures, as well as the oligomeric state (Papo and Shai, 2003).

Defensins, which contain 29-42 residues, are known to exist in solution as dimers, such as HNP-1 (White *et al.*, 1995), or monomers, such as king penguin sphenisin-2 (Landon *et al.*, 2004). These structures are larger, compared to SMAP-29, which has less amino acid residues. Therefore, defensins were hypothesised to be bacteriostatic against Gram-negative bacteria, because of the difficulties them in disrupting the bacterial membrane. Further experimental work is required to confirm this hypothesis.

Chapter 6
Conclusions and Recommendations

6.1 Overall Conclusions

This research was set out with three objectives. The first objective was to extract and purify ostrich host defence peptides from ostrich heterophils. The extraction was carried out in three steps: disruption of red blood cells with isotonic shock; disruption of heterophils with a blender; and acid extraction of host defence peptides from the heterophils granules with 10% acetic acid. It successfully yielded a crude extract that showed antimicrobial properties against Gram-negative bacteria, Gram-positive bacteria and yeast. Further purification of this crude extract was carried out in two steps, involving cationic exchange chromatography with a weak cationic resin and RP-HPLC with a step gradient of 21-29% acetonitrile and 0.01%TFA. Four ostrich host defence peptides, named ostricacins-1-4 (Osp-1-4), were successfully purified. They all displayed antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria, and Osp-2 displayed antimicrobial activity against yeast as well.

The mass spectrometry and the N-terminal sequencing analysis of these four ostricacins indicated that they had molecular weight ranging 4000-5000 Da, and they were composed of 36-42 amino acid residues. The results of these analyses suggested that these ostricacins could be classified as β-defensins, a sub-family of host defence peptides that has disulphide bridges formation between six cysteines residues. The cysteines linkages of β-defensins are Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6. This result was consistent with a hypothesis suggesting that avian host defence peptides were mainly composed of β-defensin family. There are two other types of vertebrate defensins, α- and θ-defensins, which are found only in mammals. Analysis of the amino acid composition of the ostricacins showed that they were mainly composed of arginines, lysines, glycines and cysteines. Arginines and lysines are basic amino acid residues commonly found in β-defensins, which make them positively charged. Positive charge and non-polarity are important features for the antimicrobial properties of β-defensins, particularly for the interaction with the negatively-charged bacterial

membrane. In addition, the cysteines and glycines were believed to play an important role in the structure of β-defensins, because the six cysteines and two of the glycines (Gly^{13} and Gly^{28}) were conserved amongst the avian β-defensins peptides. These eight residues were known as the "β-defensin core motif".

An evolutionary analysis, based on the alignment of the ostricacins amino acids sequences with other avian and mammalian β-defensins, revealed clustering between the avian and mammalian β-defensins, which indicated that β-defensins genes would have been around before the mammalian and avian lines diverged, approximately 310 million years ago. The evolutionary analysis also suggested that the amino acid sequence of β-defensins were highly diverged, except for the "β-defensin core motif". This was believed to be due to positive Darwinian selection that occurred at the amino acid level. Since β-defensins amino acid sequences are short, the positive Darwinian selection caused the high variation of avian and mammalian β-defensin sequences. The evolutionary analysis also indicated that vertebrate β-defensins could be categorised into three classes: the first class contained short-prepro sequences (63-64 residues) and short introns (less than 1.6 kbp); the second class contained long prepro-sequences (68-69 residues) with more than 6.5 kbp of introns; and the third class was mainly avian β-defensins. Furthermore, the avian β-defensins could be classified into two subclasses. The first subclass was comprised of avian β-defensins isolated from heterophil cells, whilst the second subclass was comprised of avian β-defensins isolated from non-heterophil cells. The ostricacins were found in both subclasses, where Osp-1 and 2 were fallen into the first subclass and Osp-3 and 4 were fallen into the second subclass. These ostricacins were closely related to the chicken gallinacins. Osp-1 and 2 were also closely related to the turkey heterophil peptides (THP-1 and 2).

The second objective was to investigate the factors affecting antimicrobial activity of ostricacins against Gram-negative bacteria and Gram-positive bacteria. The investigation was carried out using Osp-1 and Osp-2, because these two ostricacins displayed the most potency against both types of bacteria. Both ostricacins were purified with a semi-prep RP-HPLC column.

The first factor investigated was cationic ions, which included monovalent cations (Na^+ and K^+) and divalent cations (Mg^{2+} and Ca^{2+}). Both ostricacins were tested using a radial diffusion assay, which contained increasing concentrations of the cationic ions.

The results suggested that the divalent cations affected the potency of both ostricacins more significantly than the monovalent cations in inhibiting the growth of Gram-negative bacteria. Furthermore, the potency of ostricacins against Gram-positive bacteria was significantly affected by the presence of both types of cation. This was believed to be due to the presence of divalent cations around the LPS of Gram-negative bacteria, whereas these cations would not be present around Gram-positive bacterial membrane. This result agreed with the literature review, which suggested that defensins are affected by cationic ions.

The second factor investigated was temperature changes. Both ostricacins were heated at different temperatures for 30 minutes. The result indicated that the ostricacins were relatively heat stable peptides, because the potency against Gram-negative bacteria was retained when heated between 30-70°C. There was a slight decrease in the activity after the ostricacins were heated at 90°C and after autoclaving. The potency of Osp-1 against Gram-positive bacteria also demonstrated the same attribute. However, the potency of Osp-2 against Gram-positive bacteria declined after being heated at 30, 50, 70 and 90°C as well as after autoclaving. These effects were possibly caused by the loss of disulfide bridges and loss of other amino acid residues that contribute to the overall molecule heat stability. In comparison to Osp-1, the potency of Osp-2 always showed a more significant decline in the presence of cationic ions and temperature changes.

The last objective was to investigate the modes of action of the ostricacins against Gram-negative bacteria. Gram-negative bacteria have been commonly used in investigations of the mode of actions of host defence peptides. Host defence peptides are known to have two mechanisms against the bacteria, as described in the literature review. The first mechanism is membrane disruptive mechanism, where the peptides cause disruption of bacterial membrane, leading to cell leakages and cell death. The second mechanism is non-membrane disruptive mechanisms, where the peptides translocate across the membrane into the bacterial cytoplasm. In the cytoplasm, the peptides interact with intracellular components of the bacteria, such as DNA, RNA and enzymes, which inhibit the synthesis of DNA and RNA, and also enzymatic activities, leading to the inhibition of bacterial growth.

Osp-1 and Osp-2 were assayed using a series of fluorescence assays developed by Dr Hancock's research group. They were compared with SMAP-29 and HNP-1, a sheep cathelicidin peptide and a human α-defensin peptide, respectively. The three

fluorescence assays showed that affinity to LPS was the key to membrane disruptive mechanisms. SMAP-29, which demonstrated the strongest affinity to LPS, showed the ability to disrupt the Gram-negative outer membrane and cause depolarisation of the cytoplasmic membrane, whereas HNP-1, which demonstrated the weakest affinity to LPS, showed inability to disrupt the outer membrane and depolarise the cytoplasmic membrane. The ostricacins showed a moderate affinity to LPS. Moreover, the outer membrane permeabilisation assay and the cytoplasmic depolarisation assay indicated that they caused partial disruption of both membranes.

These three assays also suggested that the affinity to LPS was believed to be depended on the overall net positive charge with SMAP-29 has the highest overall net positive charge of +9, whilst the ostricacins and HNP-1 have a lower overall net positive charge of +4 and +3, respectively. In addition to the overall net positive charge, it was believed that the secondary structure of host defence peptides affected the ability of the peptides to disrupt the outer and cytoplasmic membrane of Gram-negative bacteria.

Furthermore, the ostricacins were found to be bacteriostatic against Gram-negative bacteria. The ability to inhibit bacterial growth was believed to be due to interaction between the ostricacins and the bacterial intracellular components, as show in the DNA gel electrophoresis. Neither ostricacins were bactericidal, as shown in the kill curve experiment, because they could not inhibit the colony formation potential of Gram-negative bacteria.

6.2 Recommendations for Future Work

This research on ostrich host defence peptides has yielded a great deal of useful knowledge regarding avian host defence peptides and their characteristics. It also revealed a number of new research areas. Future work could involve fundamental research that includes the following topics:

1. Purification of more host defence peptides from ostrich heterophils. Although ostrich host defence peptides have been purified with the combination of cationic exchange chromatography and RP-HPLC, further investigation using other approaches, such as gel filtration and ultrafiltration, could be carried out to purify other ostrich host defence peptides or to improve the purification yield.

2. Further investigation into the effect of pH on the antimicrobial activity of the ostricacins. In the present study, the effect of pH using the radial diffusion could not be carried out, because the pH of the agar made from the phosphate buffer was highly variable after sterilisation. There are other buffers, such as 4-morpholinoethanesulfonic acid (MES buffer), that can be used to avoid the change in pH after sterilisation.

3. Investigation of the morphological changes of bacteria when they are exposed to the ostricacins. The fluorescence assays indicated that the ostricacins caused partial disruption of Gram-negative bacterial membrane. It would be useful to know whether these peptides cause morphological change in bacteria using advance microscopy, such as the transmission electron microscope and the atomic force microscope.

4. Further testing to expand ostricacins' antimicrobial activity spectrum is recommended in order to find out the spectrum on a particular group, e.g. Gram-negative bacteria.

5. Investigation of the synergistic effects between ostrich host defence peptides as well as between the peptides and traditional antibiotics. The synergistic effect with the antibiotics may provide a solution to a growing problem of resistance pathogens. It is believed that in mammalian neutrophils, host defence peptides do not act individually, but instead, they act synergistically with other antimicrobial components, such as lactoferrin and lysozyme.

6. Investigation of the immunological properties of the ostricacins will broaden the understanding of the functional roles of these peptides in the innate immune response, such as chemotaxis and wound healing, and the linkages to adaptive immune response.

In addition to the above fundamental research, there is potential for further commercial studies relating to the ostricacins, which including the following topics:

1. As described in the literature review, ostrich host defence peptides could be added to existing topical products. Ostrich topical products, such as ostrich oil and cream, have been used in moisturisers and as remedies for arthritis, eczema, burns, abrasions, sore joints, colitis and psoriasis. The antimicrobial and immunological properties of ostricacins will increase the value of these products. A pilot-scale and a

large-scale production of ostricacins from local meat producers could be developed in New Zealand, providing benefit to the local meat producers.

2. The antiviral properties of avian host defence peptides are significant in today's world, because of the growing problem of avian disease, such as the Bird Flu. Knowledge of the avian immune response might help to stop the spread of avian diseases from birds to humans. Hence, an investigation of the antiviral activity of ostricacins would be very useful.

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Appendix 1

Raw Data and Calculations from Investigation of Antagonist Factors Affecting Osp-1 and 2

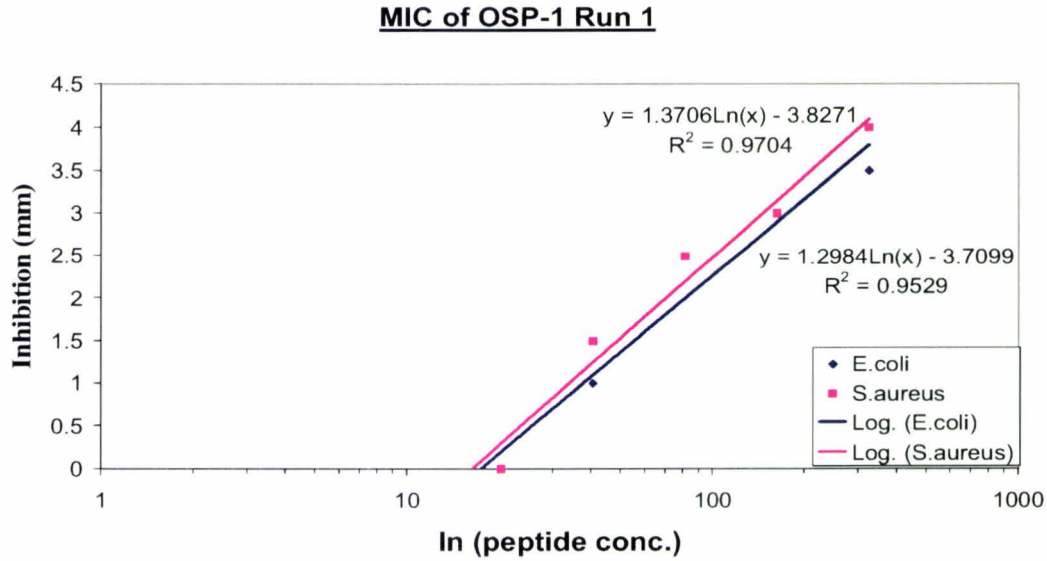
1.1 Example Calculations of MIC

The raw data of radial diffusion plate assay of Osp-1 with 10mM cationic ions is shown below.

MIC for P-19

Sample Dilution	Concentration (μg/ml)	Average Inhibition (mm)	
		E. coli	*S. aureus*
1	325.00	3.5	4
1/2	162.50	3	3
1/4	81.25	2.5	2.5
1/8	40.63	1	1.5
1/16	20.31	0	0

From this data, a graph of the average inhibition against log peptide concentration was plotted and shown below.



The MICs of Osp-1 against *E. coli* O157:H7 and *S. aureus* 1056MRSA were calculated using Equation (1) (Chapter 2, Section 2.2.8).

$$MIC_{E.coli} = e^{\frac{-Y\,\text{int}}{gradient}} = e^{\frac{-(-3.7099)}{1.2984}} = e^{2.857} \xrightarrow[\text{(inv.ln)}]{} MIC = 17.41\,{}^{\mu g}/_{ml}$$

$$MIC_{S.aureus} = e^{\frac{-Y\,\text{int}}{gradient}} = e^{\frac{-(-3.8271)}{1.3706}} = e^{2.792} \xrightarrow[\text{(inv.ln)}]{} MIC = 16.32\,{}^{\mu g}/_{ml}$$

Using Equation (1), the MICs were calculated for every run of radial diffusion plate assays, investigating the effects of cationic ions and temperatures. The raw data of each cationic ion and the temperature effects are shown in the following tables.

Effects of Na^+ on ostricacins' antimicrobial activity (μg/ml) against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

[NaCl]	Run	Microbe	OSP-1	OSP-2	Microbe	OSP-1	OSP-2
0	1	*E. coli*	1.39	1.03	*S. aureus*	1.63	2.04
0	2	*E. coli*	2.18	1.94	*S. aureus*	3.32	2.46
0	3	*E. coli*	1.79	1.49	*S. aureus*	2.48	2.25
10	1	*E. coli*	17.41	49.52	*S. aureus*	16.32	55.65
10	2	*E. coli*	15.90	49.52	*S. aureus*	16.50	55.65
10	3	*E. coli*	16.66	49.52	*S. aureus*	16.41	55.65
50	1	*E. coli*	20.31	64.65	*S. aureus*	18.81	61.07
50	2	*E. coli*	18.81	67.81	*S. aureus*	18.72	64.58
50	3	*E. coli*	19.56	66.23	*S. aureus*	18.77	62.83
100	1	*E. coli*	23.91	107.64	*S. aureus*	27.19	116.25
100	2	*E. coli*	22.28	116.25	*S. aureus*	38.14	116.25
100	3	*E. coli*	23.10	111.95	*S. aureus*	32.67	116.25
200	1	*E. coli*	81.25	232.50	*S. aureus*	78.61	232.50
200	2	*E. coli*	72.38	232.50	*S. aureus*	75.23	232.50
200	3	*E. coli*	76.82	232.50	*S. aureus*	76.92	232.50
500	1	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A
500	2	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A
500	3	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A

Effects of K^+ on ostricacins' antimicrobial activity (μg/ml) against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

[KCl]	Rep.	Microbe	OSP-1	OSP-2	Microbe	OSP-1	OSP-2
0	1	*E. coli*	1.39	1.03	*S. aureus*	1.63	2.04
0	2	*E. coli*	2.18	1.94	*S. aureus*	3.32	2.46
0	3	*E. coli*	1.79	1.49	*S. aureus*	2.48	2.25
10	1	*E. coli*	14.66	49.52	*S. aureus*	15.66	55.65
10	2	*E. coli*	15.90	49.52	*S. aureus*	17.08	55.65
10	3	*E. coli*	15.28	49.52	*S. aureus*	16.37	55.65
50	1	*E. coli*	16.89	58.14	*S. aureus*	18.40	61.07
50	2	*E. coli*	16.89	61.04	*S. aureus*	18.40	49.52
50	3	*E. coli*	16.89	59.59	*S. aureus*	18.40	55.30
100	1	*E. coli*	20.31	107.64	*S. aureus*	22.15	103.56
100	2	*E. coli*	22.15	107.64	*S. aureus*	20.31	116.25
100	3	*E. coli*	21.23	107.64	*S. aureus*	21.23	109.91
200	1	*E. coli*	43.88	232.50	*S. aureus*	77.57	N/A
200	2	*E. coli*	37.44	232.50	*S. aureus*	81.26	N/A
200	3	*E. coli*	40.66	232.50	*S. aureus*	79.42	N/A
500	1	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A
500	2	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A
500	3	*E. coli*	N/A	N/A	*S. aureus*	N/A	N/A

Effects of Mg^{2+} on ostricacins' antimicrobial activity (μg/ml) against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

[MgCl2]	Rep.	Microbe	OSP-1	OSP-2	Microbe	OSP-1	OSP-2
0	1	*E. coli*	1.39	1.03	*S. aureus*	1.63	2.04
0	2	*E. coli*	2.18	1.94	*S. aureus*	3.32	2.46
0	3	*E. coli*	1.79	1.49	*S. aureus*	2.48	2.25
2	1	*E. coli*	35.11	116.25	*S. aureus*	18.95	50.23
2	2	*E. coli*	37.44	116.25	*S. aureus*	17.68	48.88
2	3	*E. coli*	36.28	116.25	*S. aureus*	18.32	49.56
5	1	*E. coli*	72.38	N/A	*S. aureus*	21.85	53.57
5	2	*E. coli*	70.73	N/A	*S. aureus*	23.04	53.57
5	3	*E. coli*	71.56	N/A	*S. aureus*	22.45	53.57
10	1	*E. coli*	N/A	N/A	*S. aureus*	31.92	61.07
10	2	*E. coli*	N/A	N/A	*S. aureus*	31.92	61.07
10	3	*E. coli*	N/A	N/A	*S. aureus*	31.92	61.07

Effects of Ca^{2+} on ostricacins' antimicrobial activity (μg/ml) against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

[CaCl2]	Rep.	Microbe	OSP-1	OSP-2	Microbe	OSP-1	OSP-2
0	1	*E. coli*	1.39	1.03	*S. aureus*	1.63	2.04
0	2	*E. coli*	2.18	1.94	*S. aureus*	3.32	2.46
0	3	*E. coli*	1.79	1.49	*S. aureus*	2.48	2.25
2	1	*E. coli*	14.66	40.01	*S. aureus*	17.55	44.82
2	2	*E. coli*	18.95	35.43	*S. aureus*	18.81	49.52
2	3	*E. coli*	16.81	37.72	*S. aureus*	18.18	47.17
5	1	*E. coli*	15.66	69.11	*S. aureus*	18.62	76.11
5	2	*E. coli*	25.59	53.57	*S. aureus*	24.15	61.07
5	3	*E. coli*	20.63	61.34	*S. aureus*	21.39	68.59
10	1	*E. coli*	27.71	108.73	*S. aureus*	29.69	151.49
10	2	*E. coli*	20.31	116.25	*S. aureus*	25.22	130.49
10	3	*E. coli*	35.11	101.20	*S. aureus*	34.16	232.48

Effects of temperature on ostricacins' antimicrobial activity (μg/ml) against *E. coli* O157:H7 and *S. aureus* 1056MRSA.

Temp.	Rep.	Microbe	OSP-1	OSP-2	Microbe	OSP-1	OSP-2
Normal	1	*E. coli*	1.39	1.03	*S. aureus*	1.63	2.04
Normal	2	*E. coli*	2.18	1.94	*S. aureus*	3.32	2.46
Normal	3	*E. coli*	1.79	1.49	*S. aureus*	2.48	2.25
30	1	*E. coli*	5.82	4.76	*S. aureus*	6.01	12.05
30	2	*E. coli*	4.76	5.30	*S. aureus*	6.48	11.40
30	3	*E. coli*	5.29	5.03	*S. aureus*	6.25	11.73
50	1	*E. coli*	6.48	5.16	*S. aureus*	6.38	13.57
50	2	*E. coli*	6.05	6.11	*S. aureus*	5.40	12.76
50	3	*E. coli*	6.27	5.64	*S. aureus*	5.89	13.17
70	1	*E. coli*	5.48	6.01	*S. aureus*	6.48	14.01
70	2	*E. coli*	5.48	6.01	*S. aureus*	6.48	14.01
70	3	*E. coli*	5.48	6.01	*S. aureus*	6.48	14.01
90	1	*E. coli*	11.01	11.09	*S. aureus*	12.10	12.76
90	2	*E. coli*	11.01	11.09	*S. aureus*	12.10	12.76
90	3	*E. coli*	11.01	11.09	*S. aureus*	12.10	12.76
121	1	*E. coli*	9.59	12.76	*S. aureus*	11.01	11.75
121	2	*E. coli*	11.01	12.38	*S. aureus*	11.23	11.40
121	3	*E. coli*	10.30	12.57	*S. aureus*	11.12	11.58

1.2 Calculation of the Average and Confidence Intervals of MIC from the Raw Data

The average and the standard deviation of the MIC raw data shown in the previous subsection were calculated using "AVERAGE" and " STDEV" function in Microsoft Excel program. In addition, 95% confidence interval was determined using "CONFIDENCE" function in Microsoft Excel program, where the alpha value was set to 0.05. The 95% confidence interval values were used to plot the histograms in Chapter 4. The results of these statistical calculation carried out using Microsoft Excel are shown in the following tables

NaCl effect on MIC (μg/ml)

Microbe	[NaCl]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
E. coli	0	1.79	0.40	0.77	1.49	0.46	0.89
E. coli	10	16.66	0.76	0.37	49.52	0.00	0.00
E. coli	50	19.56	0.75	0.34	66.23	1.58	0.38
E. coli	100	23.10	0.82	0.33	111.95	4.31	0.80
E. coli	200	76.82	4.44	1.00	232.50	0.00	0.00
E. coli	500	N/A	N/A	N/A	N/A	N/A	N/A

NaCl effect on MIC (μg/ml)

Microbe	[NaCl]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
S. aureus	0	2.48	0.85	1.17	2.25	0.21	0.29
S. aureus	10	16.41	0.09	0.04	55.65	0.00	0.00
S. aureus	50	18.77	0.05	0.02	62.83	1.76	0.44
S. aureus	100	32.67	5.48	1.90	116.25	0.00	0.00
S. aureus	200	76.92	1.69	0.38	232.50	0.00	0.00
S. aureus	500	N/A	N/A	N/A	N/A	N/A	N/A

KCl effect on MIC (μg/ml)

Microbe	[KCl]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
E. coli	0	1.79	0.40	0.77	1.49	0.46	0.89
E. coli	10	15.28	0.62	0.31	49.52	0.00	0.00
E. coli	50	16.89	0.00	0.00	59.59	1.45	0.37
E. coli	100	21.23	0.92	0.39	107.64	0.00	0.00
E. coli	200	40.66	3.22	1.00	232.50	0.00	0.00
E. coli	500	N/A	N/A	N/A	N/A	N/A	N/A

KCl effect on MIC (μg/ml)

Microbe	[KCl]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
S. aureus	0	2.48	0.85	1.17	2.25	0.21	0.29
S. aureus	10	16.37	0.71	0.35	55.65	0.00	0.00
S. aureus	50	18.40	0.00	0.00	55.30	5.78	1.53
S. aureus	100	21.23	0.92	0.39	109.91	6.35	1.19
S. aureus	200	79.42	1.85	0.41	N/A	N/A	N/A
S. aureus	500	N/A	N/A	N/A	N/A	N/A	N/A

$MgCl_2$ effect on MIC (μg/ml)

Microbe	[MgCl2]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
E. coli	0	1.79	0.40	0.77	1.49	0.46	0.89
E. coli	2	36.28	1.17	0.38	116.25	2.29	0.42
E. coli	5	71.56	0.83	0.19	N/A	N/A	N/A
E. coli	10	N/A	N/A	N/A	N/A	N/A	N/A

$MgCl_2$ effect on MIC (μg/ml)

Microbe	[MgCl2]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
S. aureus	0	2.48	0.85	1.17	2.25	0.21	0.29
S. aureus	2	18.32	0.64	0.29	49.56	0.68	0.19
S. aureus	5	22.45	0.60	0.25	53.57	0.00	0.00
S. aureus	10	31.92	0.00	0.00	61.07	0.00	0.00

$CaCl_2$ effect on MIC (μg/ml)

Microbe	[CaCl2]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
E. coli	0	1.79	0.40	0.77	1.49	0.46	0.89
E. coli	2	16.81	2.15	1.05	37.72	2.29	0.00
E. coli	5	20.63	4.97	2.18	61.34	7.77	1.95
E. coli	10	27.71	7.40	2.79	108.73	7.53	1.42

$CaCl_2$ effect on MIC

Microbe	[CaCl2]	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
S. aureus	0	2.48	0.85	1.17	2.25	0.21	0.29
S. aureus	2	18.18	0.63	0.29	47.17	2.35	0.67
S. aureus	5	21.39	2.77	1.18	68.59	7.52	1.79
S. aureus	10	29.69	4.47	1.63	140.99	14.85	2.46

Temperature effect on MIC (μg/ml)

Microbe	Temp.	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
E. coli	Normal	1.79	0.40	0.77	1.49	0.46	0.89
E. coli	30	5.29	0.53	0.46	5.03	0.27	0.24
E. coli	50	6.27	0.22	0.17	5.64	0.48	0.42
E. coli	70	5.48	0.00	0.00	6.01	0.00	0.00
E. coli	90	11.01	0.00	0.00	11.09	0.00	0.00
E. coli	121	10.30	0.71	0.44	12.57	0.19	0.11

Temperature effect on MIC (μg/ml)

Microbe	Temp.	OSP-1	St. Dev	C.I. (95%)	OSP-2	St. Dev	C.I. (95%)
S. aureus	Normal	2.48	0.85	1.17	2.25	0.21	0.29
S. aureus	30	6.25	0.24	0.19	11.73	0.33	0.19
S. aureus	50	5.89	0.49	0.43	13.17	0.41	0.22
S. aureus	70	6.48	0.00	0.00	14.01	0.00	0.00
S. aureus	90	12.10	0.00	0.00	12.76	0.00	0.00
S. aureus	121	11.12	0.11	0.07	11.58	0.18	0.10

1.3 Analysis of Variance (ANOVA) of MIC Data from Different Conditions

The ANOVA was done using a two-way ANOVA function of the MINITAB program with 95% confidence interval. The y-variate was ln MIC of the peptide, whilst the row and column factors were the concentration of the cations/temperature conditions and the number of repeats, respectively. The ANOVA calculation was carried out for all the investigated factors (monovalent cations, divalent cations and temperatures). An example of ANOVA calculation on MICs of Osp-1 against *E. coli* that was affected by Na^+ and the number of runs are shown below.

Two-way ANOVA: Osp-1 on *E. coli* versus [NaCl], Repeats

Source	DF	SS	MS	F	P
[NaCl]	4	9879.86	2469.96	730.11	0.000
Repeats	2	16.18	8.09	2.39	0.153
Error	8	27.06	3.38		
Total	14	9923.10			

S = 1.839 R-Sq = 99.73% R-Sq(adj) = 99.52%

The ANOVA results are summarised in the following tables.

Summary of ANOVA Results against *E. coli* O157:H7

Peptide	Condition	Factor	m.s. factor	d.f. factor	p-value
OSP-1	Na^+	Conc.	2469.96	4	0.000
		Repeats	8.09	2	0.153
	K^+	Conc.	591.43	4	0.000
		Repeats	0.33	2	0.892
	Mg^+	Conc.	3651.05	2	0.000
		Repeats	0.18	2	0.843
	Ca^+	Conc.	359.05	3	0.000
		Repeats	13.92	2	0.500
	Temperature	Conc.	35.60	5	0.000
		Repeats	0.02	2	0.895
OSP-2	Na^+	Conc.	23099.10	4	0.000
		Repeats	8.00	2	0.149
	K^+	Conc.	23261.50	4	0.000
		Repeats	0.70	2	0.221
	Mg^+	Conc.	19755.90	1	0.000
		Repeats	0.10	2	0.500
	Ca^+	Conc.	6060.26	3	0.000
		Repeats	19.15	2	0.600
	Temperature	Conc.	50.92	5	0.000
		Repeats	0.17	2	0.152

Summary of ANOVA Results against *S. aureus* 1056MRSA

Peptide	Condition	Factor	m.s. factor	d.f. factor	p-value
OSP-1	Na^+	Conc.	2456.61	4	0.000
		Repeats	4.37	2	0.572
	K^+	Conc.	2675.61	4	0.000
		Repeats	1.23	2	0.321
	Mg^+	Conc.	452.12	3	0.000
		Repeats	0.16	2	0.705
	Ca^+	Conc.	389.11	3	0.000
		Repeats	4.76	2	0.581
	Temperature	Conc.	38.77	5	0.000
		Repeats	0.08	2	0.659
OSP-2	Na^+	Conc.	22903.40	4	0.000
		Repeats	0.80	2	0.321
	K^+	Conc.	5795.41	4	0.000
		Repeats	0.15	2	0.994
	Mg^+	Conc.	2134.09	3	0.000
		Repeats	0.05	2	0.709
	Ca^+	Conc.	15390.30	3	0.001
		Repeats	757.30	2	0.412
	Temperature	Conc.	56.54	5	0.000
		Repeats	0.08	2	0.264

Appendix 2

Replicates Results of the Investigation of Mechanisms of Actions of Ostricacins

The fluorescence assays and the kill curve experiment were done in three replicates. In this section, the results of the three replicates are shown. The raw data are not included, because there are too many data point, particularly for the outer membrane permeabilisation assay and the cytoplasmic membrane depolarisation assay. The data for these two assays were recorded every second for approximately 6.5 minutes.

2.1 Raw Data of LPS Binding Assay and Example Calculations of Fraction Fluorescence Inhibited

The raw data of LPS binding assay of Osp-1 is shown below.

[OSP-1] (μg/ml)	Florescence reading	Fraction Fluorescence inhibited
0.0	126.750	0.000
0.9	125.990	0.006
1.3	123.080	0.029
1.7	120.920	0.046
2.1	119.070	0.061
2.6	118.130	0.068
3.0	117.750	0.071
3.4	113.780	0.102
3.8	113.100	0.108
4.3	116.590	0.080
4.7	116.310	0.082
5.1	114.550	0.096
5.5	114.510	0.097

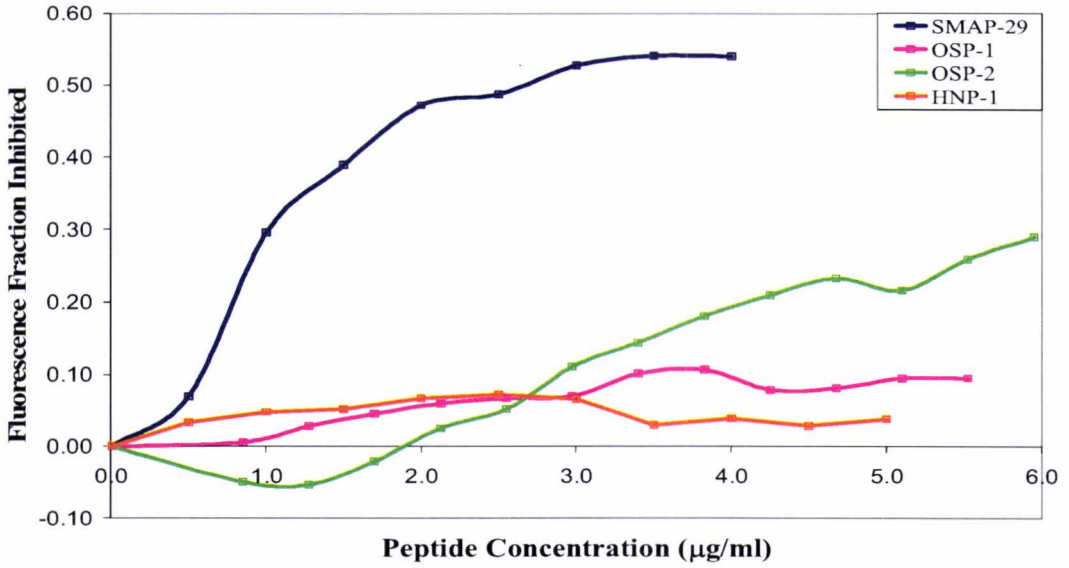
The fraction fluorescence inhibited was calculated using Equation (2) (Chapter 2, Section 2.4.1). An example of calculation is shown below, after the first addition Osp-1.

$$F_{inhib} = \frac{F_{initial} - F_n}{F_{initial}} = \frac{126.75 - 125.99}{126.75} = 0.006$$

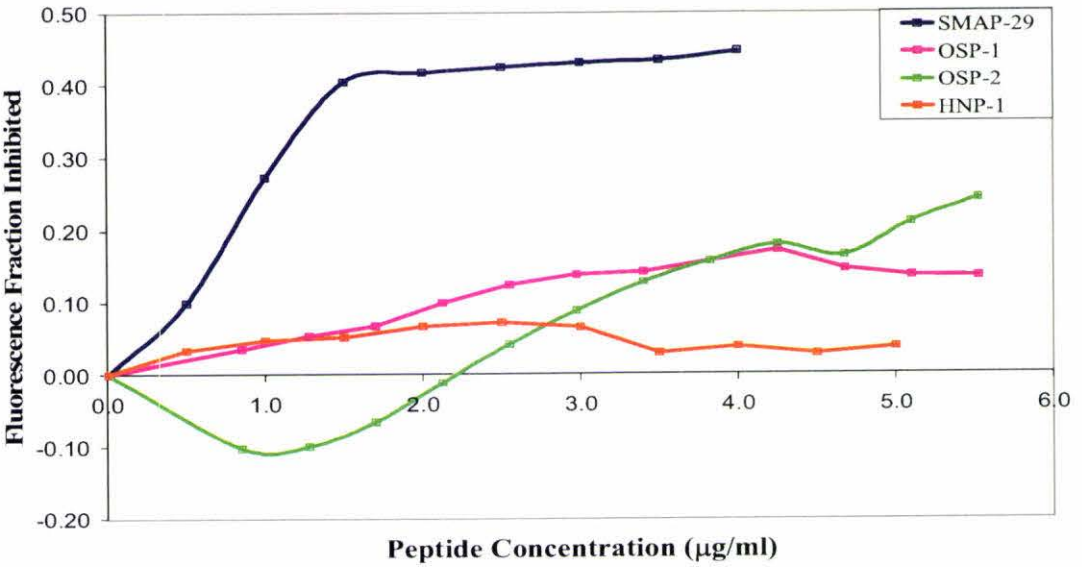
This calculation was carried out for the four assayed peptides (Osp-1, Osp-2, SMAP-29 and HNP-1). The result was then plotted as fraction fluorescence inhibited against peptide concentration. These plots from three assay replicates are shown in the following section.

2.2 Results of LPS Binding Assay

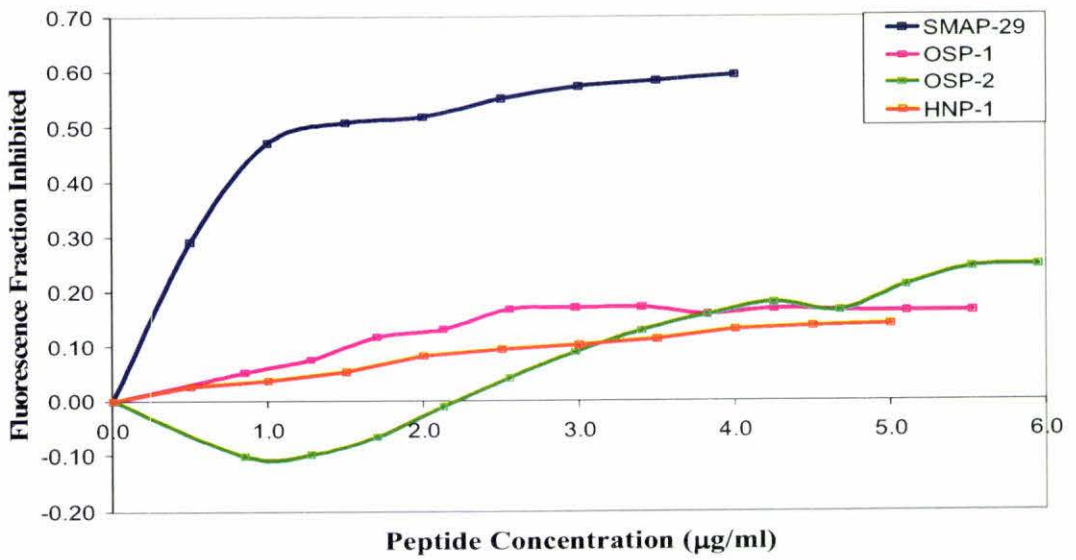
Run I



Run II

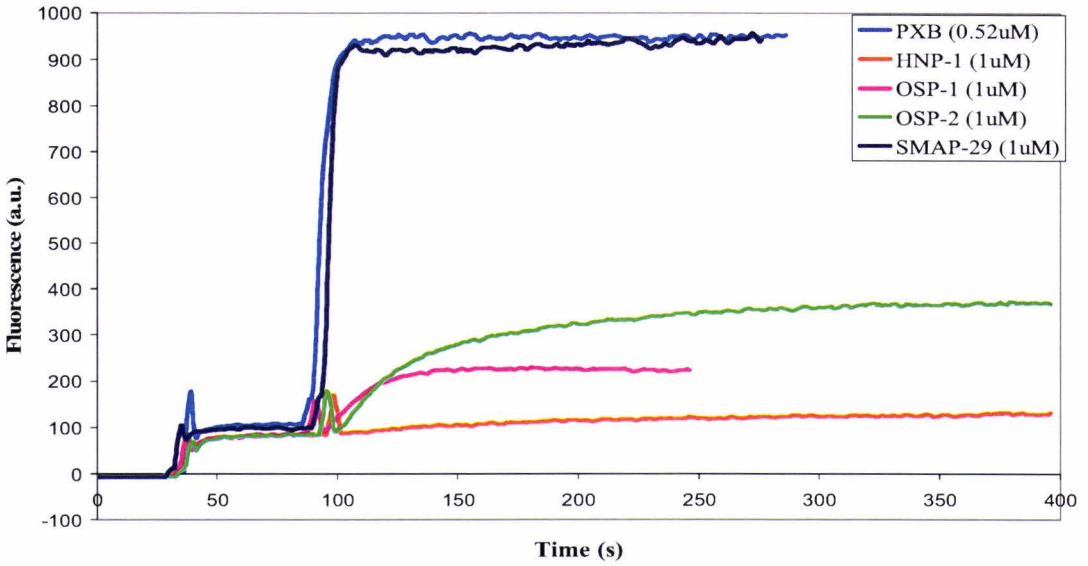


Run III

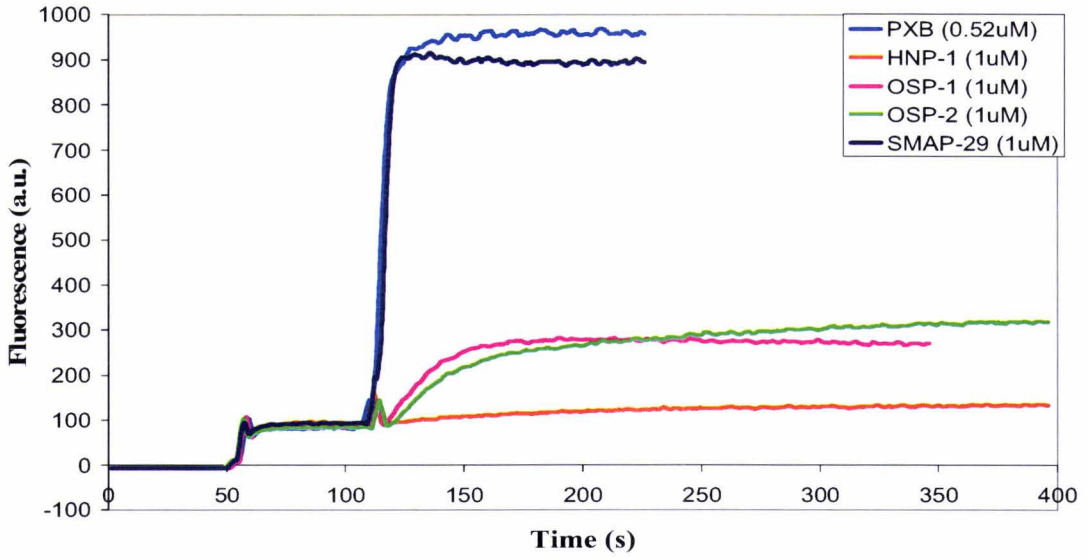


2.3 Results of Outer Membrane Permeabilisation Assay

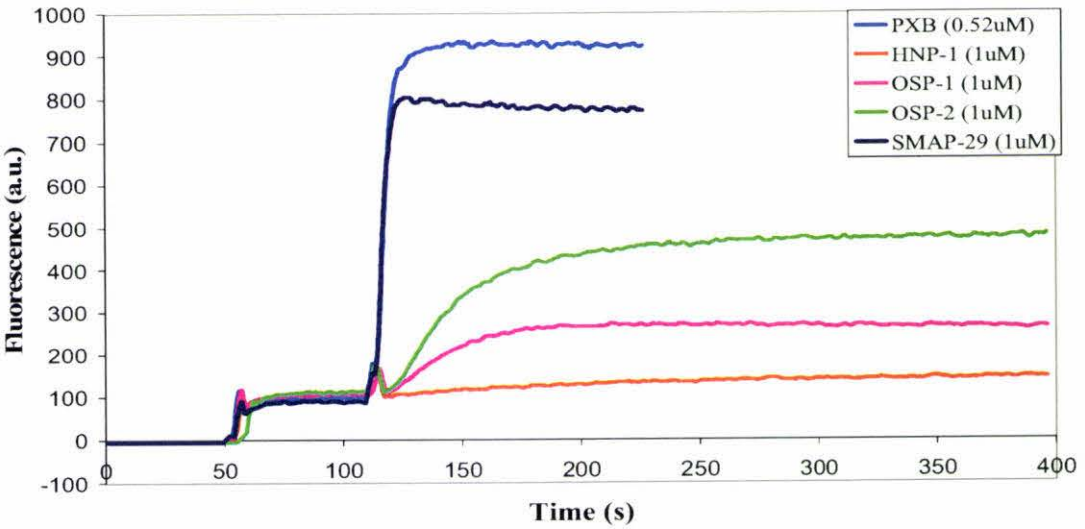
Run I



Run II

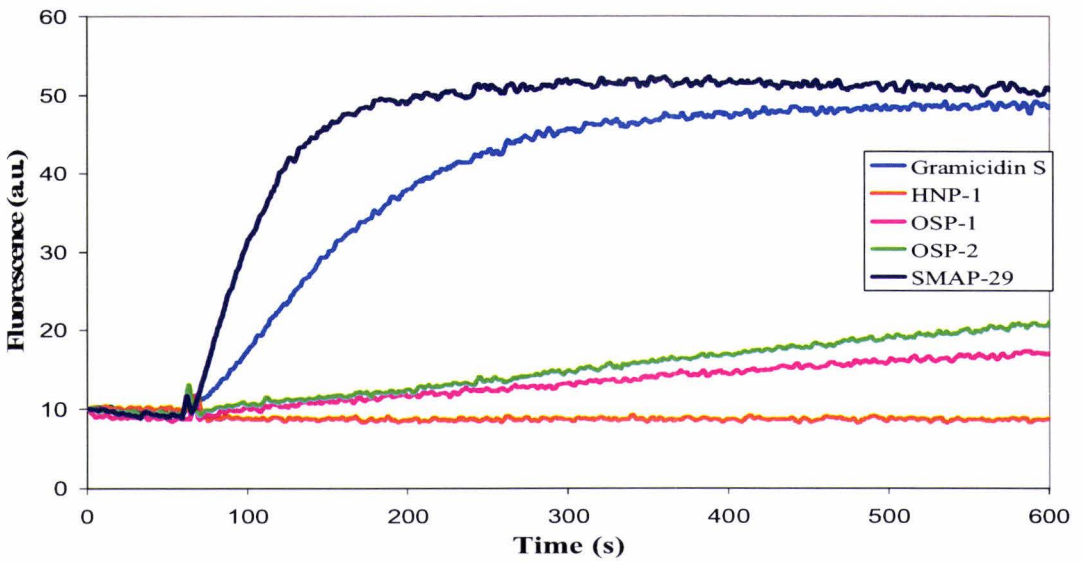


Run III

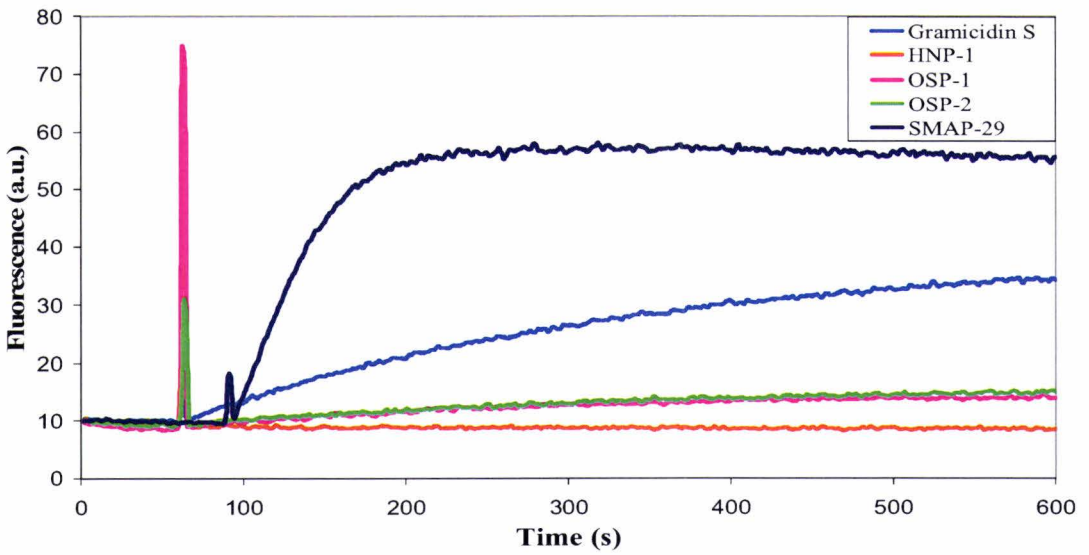

1000
900
800
700
600
500
400
300
200
100
0
-100
Fluorescence (a.u.)
0
50
100
150
200
250
300
350
400
Time (s)
PXB (0.52uM)
HNP-1 (1uM)
OSP-1 (1uM)
OSP-2 (1uM)
SMAP-29 (1uM)

2.4 Results of Cytoplasmic Membrane Depolarisation Assay

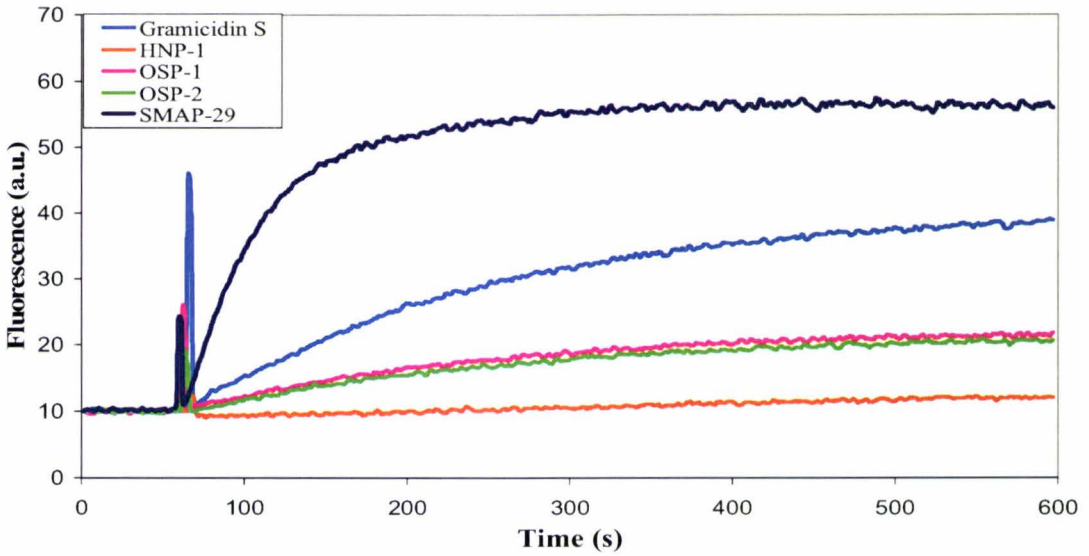
Run I



Run II



Run III

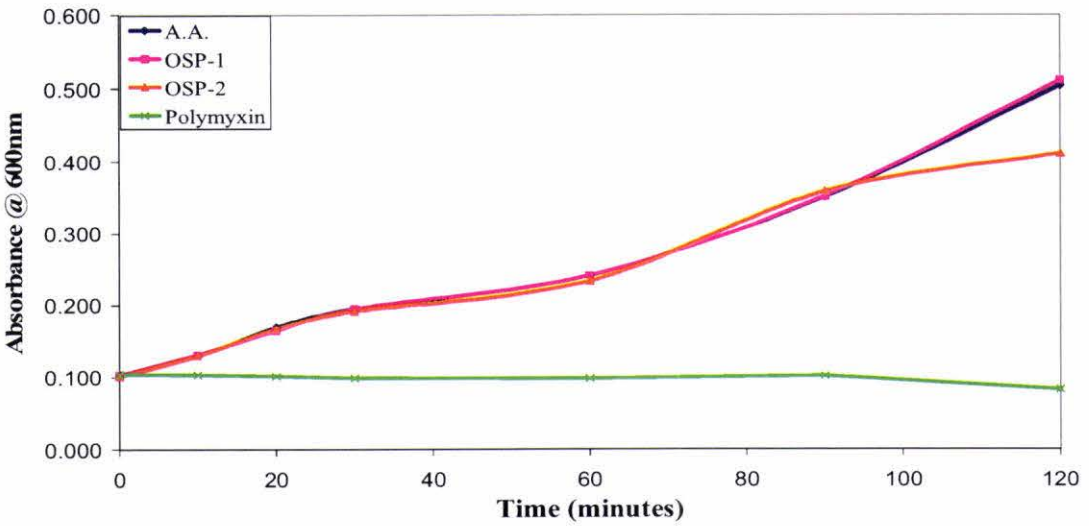

Gramicidin S
HNP-1
OSP-1
OSP-2
SMAP-29
Fluorescence (a.u.)
Time (s)
0
10
20
30
40
50
60
70
0
100
200
300
400
500
600

2.5 Results of Kill Curves on *E. coli* O111

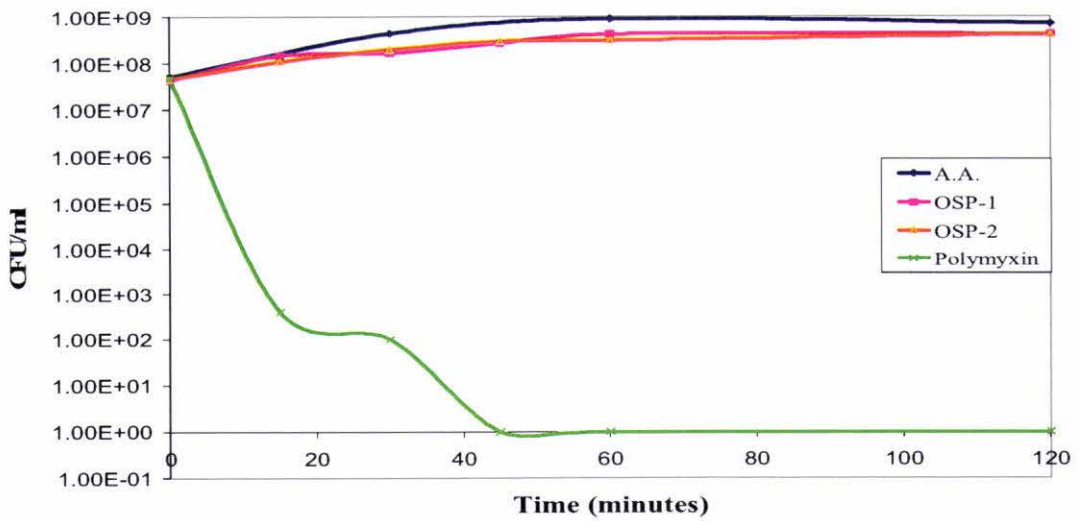
Acetic acid (A.A.) was used as the negative control and polymyxin B was used as the positive control.

Run I

Measurements of optical density

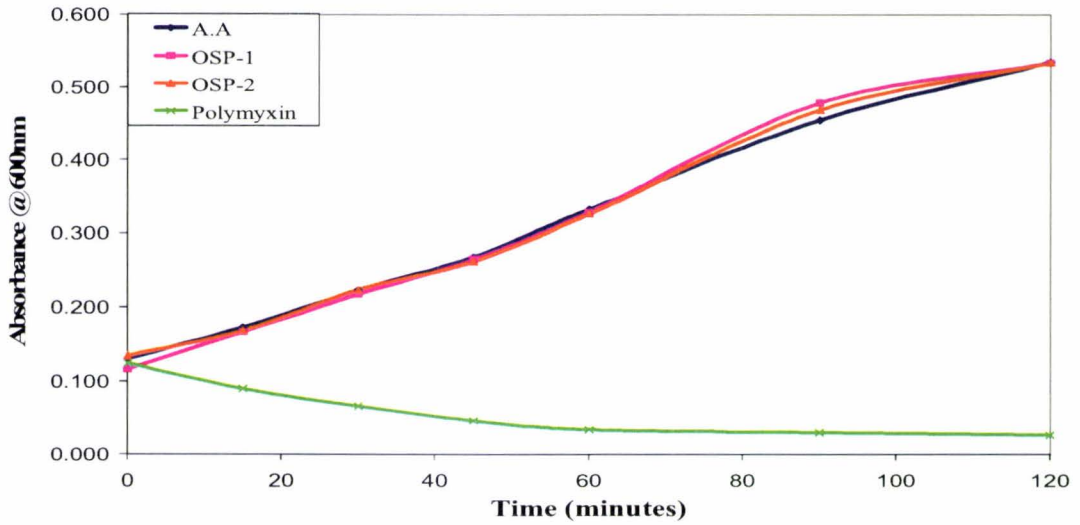


Counting bacterial colonies

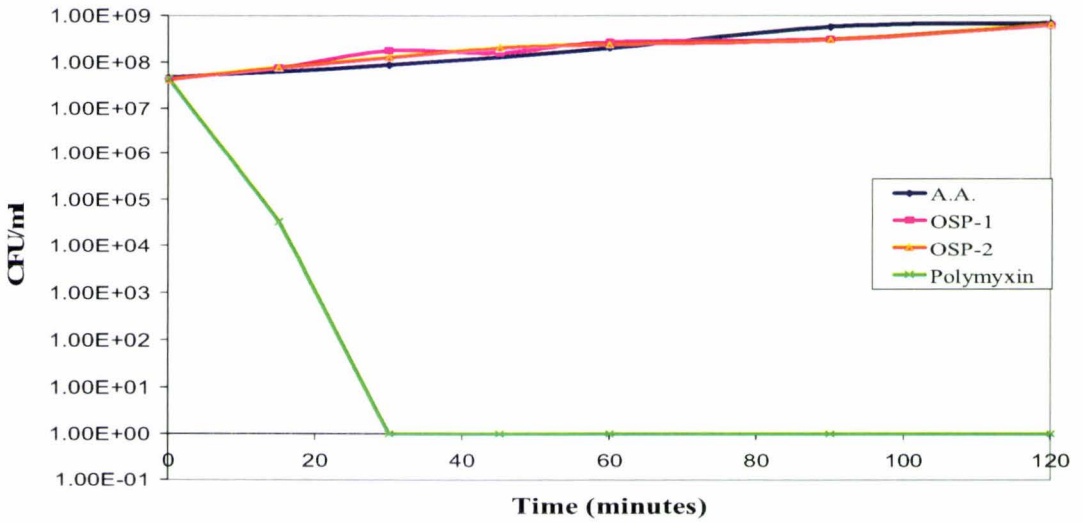


Run II

Measurements of optical density

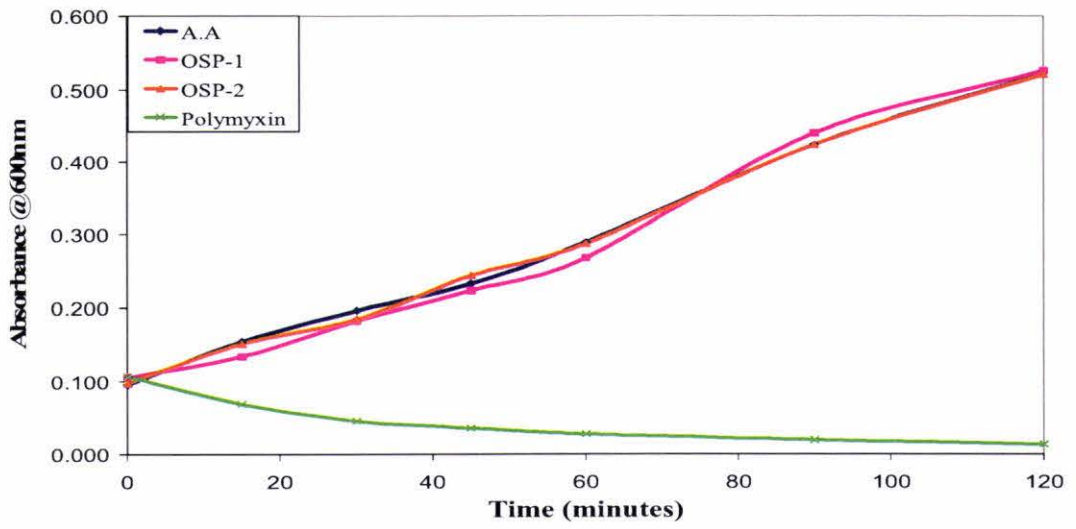


Counting bacterial colonies

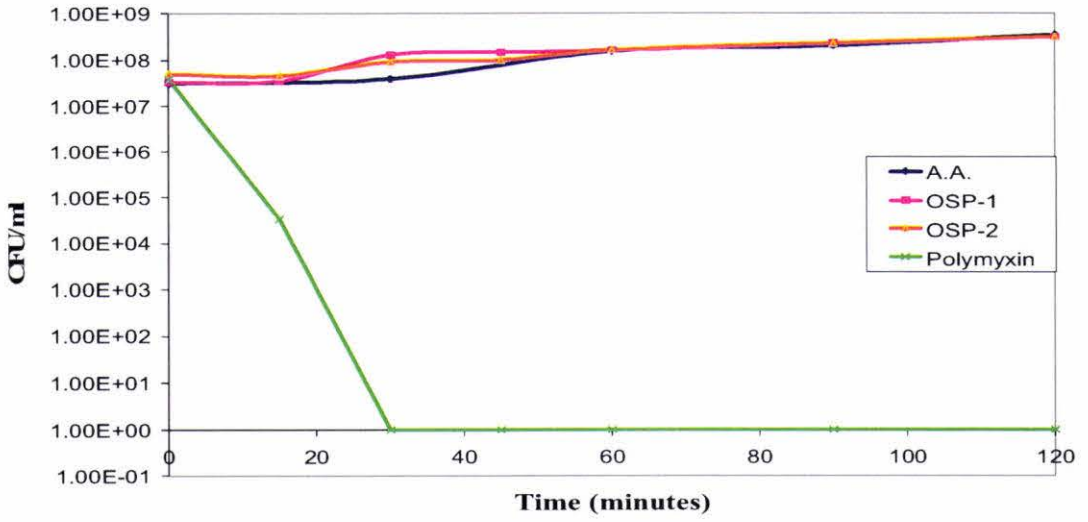


Run III

Measurements of optical density



Counting bacterial colonies



Appendix 3

List of Publications

Some of the research presented in this thesis has been published in journals and presented in conferences. There are some researches that are in preparations. These are listed below.

Journal Articles

Sugiarto, H. and Yu, P.L. (2004). Avian Antimicrobial Peptides: the Defence Role of β-Defensins. **Biochemical and Biophysical Research Communications**, **323**(3), 721-727.

Sugiarto, H. and Yu, P.L. (2006). Identification of Three Novel Ostricacins: an Update on the Phylogenetic Perspective of β-Defensins. **International Journal of Antimicrobial Agents**, **27**(3), 229-235.

Conference Proceedings

Sugiarto, H. and Yu, P.L. (2004). Evolutionary Relationship and the Multifunctional Roles of Avian β-Defensins. **International Conference on Bioinformatics**, 5th-8th September 2004, Auckland, New Zealand.

Sugiarto, H. and Yu, P.L. (2004). Isolation and Characterisation of Ostrich β-Defensins. **New Zealand Microbiological Society 49th Annual Conference**, 17th-19th November 2004, Palmerston North, New Zealand.

Articles in Preparations

Sugiarto, H. and Yu, P.L. Investigation of Factors Affecting the Antimicrobial Activity of Ostricacins-1 and 2 on *E. coli* O157:H7 and *S. aureus* 1056MRSA.

Sugiarto, H., Power, J.P.S., Hancock, R.E.W. and Yu, P.L. Investigation of Mechanisms of Actions of Ostrich β-Defensins against Gram-Negative Bacteria.

van Der Linden, D.S., **Sugiarto, H**. and Yu, P.L. Neutrophil-Derived Antimicrobial Peptides in Farm Animals.