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Antibacterial Proteins and Peptides in Nurse Shark (*Ginglymostoma Cirratum*) Peripheral Blood Leukocytes

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

ANTIBACTERIAL PROTEINS AND PEPTIDES IN NURSE SHARK (*GINGLYMOSTOMA*
CIRRATUM) PERIPHERAL BLOOD LEUKOCYTES

A dissertation submitted in partial fulfillment of the

requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Nichole Hinds Vaughan

2011

To: Dean Kenneth Furton
College of Arts and Sciences

This dissertation, written by Nichole Hinds Vaughan, and entitled Antibacterial Proteins and Peptides in Nurse Shark (*Ginglymostoma Cirratum*) Peripheral Blood Leukocytes, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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This dissertation of Nichole Hinds Vaughan is approved.

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Florida International University, 2011

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DEDICATION

I dedicate this dissertation to my mom and dad, who taught me that anything was possible if I believed in myself. You are both sorely missed.

To my husband, Robert, and my daughter, Maya, you are my light!

ACKNOWLEDGMENTS

There are many people who have contributed in one way or another to the successful completion of this study. While it would be impossible to list everyone individually, I would like to say a big thank you, from the bottom of my heart, to everyone who assisted me along the way. In particular, I wish to thank Dr. Sylvia Smith for her consistent mentorship, keen scientific observations and for her expert advice. I would also like to thank Dr. Charles Bigger and the members of my committee for their patience and dedication to moving me forward through this process.

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....And last but not least, I would like to thank my sisters, who drive me crazy, while keeping me perfectly sane. Thanks for the endless babysitting and for your lifelong belief in me.

ABSTRACT OF THE DISSERTATION

ANTIBACTERIAL PROTEINS AND PEPTIDES IN NURSE SHARK (*GINGLYMOSTOMA
CIRRATUM*) PERIPHERAL BLOOD LEUKOCYTES

by

Nichole Hinds Vaughan

Florida International University, 2011

Miami, Florida

Professor Charles H. Bigger, Major Professor

In many vertebrate and invertebrate species mediators of innate immunity include antimicrobial peptides (AMPs) such as peptide fragments of histones and other proteins with previously ascribed different functions. Shark AMPs have not been described and this research examines the antibacterial activity of nurse shark (*Ginglymostoma cirratum*) peripheral blood leukocyte lysates. Screening of lysates prepared by homogenizing unstimulated peripheral blood leukocytes identified muramidase (lysozyme-like) and non-muramidase antibacterial activity. Lysates were tested for lysozyme using the lysoplate assays, and antibacterial (AB) activity was assayed for by a microdilution growth assay that was developed using *Planococcus citreus* as the target bacterium. Fractionation of crude lysates by ion exchange and affinity chromatography was followed by a combination of SDS-PAGE with LC/MS-MS and/or N-terminal sequence analysis of low molecular weight protein bands (<20 kDa). This yielded several peptides with amino acid sequence similarity to lysozyme, ubiquitin, hemoglobin, human histones H2A, H2B and H4 and to antibacterial histone fragments of

the catfish and the Asian toad. Not all peptide sequences corresponded to peptides potentially antibacterial. The correlation of a specific protein band in active lysate fractions was accomplished by employing the acid-urea gel overlay assays in which AB activity was seen as zones of growth inhibition on a lawn of *P. citreus* at a position corresponding to that of the putative AB protein band. This study is the first to describe putative AMPs in the shark and their potential role in innate immunity.

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I. INTRODUCTION

The mammalian immune system is a complex network of cells, organs, tissue, and soluble protein substances that interact to protect the body from foreign materials, recognized as non-self. This protection can take the form of an adaptive, targeted response or an innate and immediate response. Immune cells will surveil, and detect, non-native molecules and either mount an attack or call in reinforcements, or both (Klein 1997).

The innate immune system is comprised of several components: (1) physical and chemical barriers such as the skin, the low pH of the stomach, and the sweeping motion of the cilia in the airway; (2) cellular and (3) humoral factors. The cellular components consist of a variety of different cell types that include macrophage/monocytes, granulocytes such as neutrophils, basophils and eosinophils, mast cells, dendritic cells and natural killer cells and can function by direct intervention through either engulfment of the foreign particle or by indirect interaction when stimulated through subsequent secretion of soluble proteins (Kindt et al. 2007). Humoral factors include soluble proteins such as complement proteins, hydrolyzing enzymes, cytokines and the ubiquitous antimicrobial peptides (Kindt et al. 2007). Antimicrobial peptides, key mediators of innate immune function, have emerged as effective defense molecules, patrolling host borders, fighting off and killing invading pathogens, and acting as the first line of defense.

Antimicrobial peptides, which have recently been renamed as host defense peptides (Steinstraesser et al. 2009), play a significant role in angiogenesis (Huang et al. 1997; Li et al. 2000), chemotaxis (Fleischmann et al. 1985; Ichinose et al. 1996), cytokine production (Chaly et al. 2000), histamine release (Befus et al. 1999; Niyonsaba et al. 2001), lipopolysaccharide (LPS)-binding (Nagaoka et al. 2001; Nagaoka et al. 2002) and other immunomodulatory activities (Yang et al. 2002; Bals et al. 2003; Bowdish et al. 2005). However, the main function of antimicrobial peptides is to kill microbes. Collectively, these peptides exhibit a broad spectrum of activity against microbes, including gram positive and gram negative bacteria, fungi, mycoplasma, and viruses (reviewed in Boman, 1998).

Antimicrobial peptides have been isolated from a wide variety of animals. They are present in several vertebrates including mammals (Selsted et al. 1985; Zanetti et al. 1995), birds (Harwig et al. 1994; Sugiarto et al. 2004), amphibians (Zaslhoff 1987) and teleost fish, (Cole et al. 1997; Noga et al. 2003) and in invertebrates, such as insects (Boman et al. 1985; Casteels et al. 1989; Moore et al. 1996), horse-shoe crabs (Schnapp et al. 1996; Relf et al. 1999), tunicates (Lee et al. 1997) and shrimp (Destoumieux et al. 1997). They have also been documented in plants (Rinehart et al. 1990; Harrison et al. 1999; Stephens et al. 2005) and bacteria (Boman 1998, 2003; Sang et al. 2008).

To date, little is known of antimicrobial peptides in elasmobranchs, including sharks, although antimicrobial proteins such as transferrin (Davies et al. 1987) and squalamine, an aminosterol (Moore et al. 1993), have been identified in the dogfish shark. A cationic antimicrobial peptide has been described for the fermented skate, *Raja kenoeji*, from

skin extracts (Cho et al. 2005). There have also been some functional studies conducted on lysozyme-like activity using nurse shark serum (Hedayati 1992). There are many generalities shared by this class of vertebrates. Each species, however, must be examined on its own merit. Rays, sharks and other elasmobranchs display many differences in habitat, behavior and physiology. Even sharks within the same subdivision differ in some features as fundamental as the function of hematopoietic organs (Fange 1977), therefore It is unwise to use one species of sharks to wholly represent other species.

Most investigators studying the immune system of the shark focus on adaptive immune mechanisms (Greenberg et al. 1995; Ohta et al. 2002; Rumfelt et al. 2002; Dooley et al. 2005; Haines et al. 2005; Zhang et al. 2006; Flajnik et al. 2009). Research is also being conducted on innate immune mechanisms of the shark, particularly the complement system and the role and function of circulating leukocytes (Smith et al. 1989; Dodds et al. 1998; Merly et al. 2007; Shin et al. 2007; Aybar et al. 2009; Graham et al. 2009; Shin et al. 2009). The composition of shark peripheral blood differs from humans in several ways (Hyder et al. 1983). Firstly, shark serum contains relatively high concentrations of salt (0.25 M) and urea (0.35 M) and its complement proteins have certain unique features. In addition, the bulk protein in serum is not albumin, as is seen in most mammals, but rather immunoglobulin (natural antibody) which can amount to as much as 40% of total serum protein. Secondly, the cellular components of shark blood also show differences. Curiously, there has not been a published histological study using light microscopy, of nurse shark (the shark used in this study) peripheral

blood leukocytes. However, some general cellular characteristics prevail. Unlike the non-nucleated discoidal human erythrocytes, shark mature erythrocytes are nucleated ellipsoidal structures. Shark granulocytic leukocytes morphologically resemble mammalian neutrophils and eosinophils, and the mononuclear cells are similar to lymphocytes and monocyte/macrophages. The shark granulocytes and monocyte/macrophages have been shown to be phagocytic and chemotactic cells (Hyder et al. 1983; Obenauf et al. 1985). In addition, shark blood contains the thrombocyte, a cell absent from mammalian peripheral blood. Thrombocytes are believed to play a role in coagulation, similar to that of human platelets which the shark lacks. The shark lacks bone marrow (its skeleton is composed of cartilage) and it is not unusual to find immature undifferentiated cells circulating in peripheral blood (Hyder et al. 1983).

Antimicrobial peptides can be produced by a variety of cell types such as: hemocytes of the crab (Schnapp et al. 1996), cecropia moth (Steiner et al. 1981) and pacific shrimp (Patat et al. 2004), mammalian paneth cells (Mallow et al. 1996), epithelial cells (Diamond et al. 1991), and neutrophils (Befus et al. 1999); and skin mucosa of fish (Cho et al. 2002) and frogs (Zasloff 1987). To date, no investigation has been undertaken to investigate the antimicrobial proteins and/or peptides associated with circulating leukocytes in the nurse shark.

The major objective of this research is the identification and characterization of antibacterial activity of nurse shark, *Ginglymostoma cirratum*, peripheral blood leukocytes. Peripheral blood leukocytes were chosen as the cell population to study

since circulating leukocytes in other vertebrate species play an important role in the initial line of defense and are known to secrete a variety of antimicrobial peptides (Spitznagel 1984, 1990). Earlier studies showed two distinctly different types of antimicrobial activity associated with shark peripheral leukocytes: a lysozyme-like activity and a non-lysozyme-like antibacterial activity (Hinds et al. 2001).

II. BACKGROUND

In plants and animals, gene-encoded antimicrobial peptides are rapidly expressed and delivered directly to sites of microbial infiltration (Boman 1998, 2003). This rapid response, though non-specific, differs from the clonally based adaptive immune response (Burnet 1957), which is an acquired response to stimuli.

Intracellular Immune Factors

When combating an infection, the mammalian innate immune system is called upon to mount an immediate response to invading pathogenic microbes. With generation times of 20-30 min (Boman 1995), this response, once thought to be 'non-specific', can immediately mobilize phagocytic and cytotoxic cells like macrophages, neutrophils, T-cells and Natural killer (NK) cells to release signaling molecules such as interleukins (IL-1, IL-2) and tumor necrosis factor-alpha (TNF- α) (Klein 1997) and other effector molecules such as lectins (Feizi 2000), NK-lysin (Andersson et al. 1995) or nitric oxide (Fang 1997). The quick response differs from the acquired immune response, which because of its incredible specificity, may take days and even weeks for the clonal expansion of T and B lymphocytes to target specific antigens (Klein 1997).

The innate immune defense must selectively destroy or inactivate the invading pathogen, while protecting the structural integrity of the host. In order to do this, receptor mediated recognition of common molecular features differentiating host cells from foreign cells, is necessary. These pathogen-associated molecular patterns (PAMPs) can take the form, for example, of surface associated glycans, formylated

peptides, lipopolysaccharide, CpG DNA and coat proteins of viruses (Brown 2001; Girardin et al. 2002). The pattern recognition results in the delivery of effector molecules to the site of infection, such as complement activation on the microbial surface, or antigen/pathogen ingestion followed by the release of reactive oxygen products and granule contents into phagocytic vacuoles (Ganz et al. 1997). These defense mechanisms can inhibit the entry of the microbe, disruption of crucial microbial structures, sequestration of essential nutrients for microbial survival, interference with microbial metabolism, or inhibition of microbial multiplication. These mechanisms utilize inorganic compounds such as hydrogen peroxide, hydrochloric acid, hypochlorous acid, or organic compounds such as chloramines, fatty acids, peptides and proteins (Ganz et al. 1997). The molecules that perform these defense functions (some of which are discussed below) are varied in their physicochemical characteristics, their mechanism of action and their location.

It is evident that the intracellular components are exceedingly potent and are crucial to host defense. The phagocytic cells, neutrophils, monocytes and macrophages play a significant role in immune function, monitoring the body for any invading agent. Once stimulated, these phagocytes possess an impressive arsenal that includes production of bactericidal-reactive oxygen compounds, release of the contents of cytoplasmic granules into the phagosome or the exterior, secretion of cytokine and other regulatory intermediates (thereby attracting other cells to the site of infection), crossing the blood vessel endothelia, with active movement through

tissue towards source of infection, and production of bactericidal nitric oxide and its derivatives (Klein 1997).

Antimicrobial Proteins

There are several proteins that are associated with the non-oxidative response in mammalian phagocytic cells that are antimicrobial in nature, one of which is cathepsin G, a serine protease that is found in the azurophilic granules of neutrophils, and some monocytes (Odeberg et al. 1975; Senior et al. 1982; Spitznagel 1990; Kudo et al. 2009). Cathepsin G is a 26 kDa neutral enzyme that is released by activated neutrophils in humans, and plays an important role in inflammation through the hydrolysis of many different proteins, including extracellular matrix and some chemo-attractants such as TNF- α and IL-8 (Wiedow et al. 2005).

Phospholipase A2 selectively hydrolyzes phospholipids in bacterial membranes, and is a member of a large family of 'secretory' phospholipases that are produced by vertebrates and invertebrates (Dennis 1997). The family of phospholipase A2 shares several highly conserved features, including size (14-16 kDa), high disulfide bond content, a Ca²⁺-binding loop, and closely similar catalytic machinery and secondary and tertiary structure (Dennis 1997; Ganz et al. 1997). Bactericidal/permeability-increasing protein, BPI, is a highly cationic protein known for its potent and selective cytotoxic activity toward gram-negative bacteria and its high affinity for LPS (Elsbach et al. 1993).

Transferrins are non-heme, globular, iron-binding glycoproteins, found in the serum of most vertebrates; they are also present in avian egg white and in mammalian

milk (Putnam 1975; Ingram 1980). Human transferrin has an approximate molecular weight of 80-90 kDa and displays bacteriostatic activity when not fully saturated with iron. Its role when confronting a bacterial infection consists of chelating any free available iron present, making it unavailable to the invading organism (Weinberg 1974; Ingram 1980). Lactoferrin, a member of the transferrin family, is a high affinity iron-chelator in milk, and also displays bacteriostatic properties (Bullen et al. 1979; Ellison et al. 1991; Bellamy et al. 1993). Lactoferrin has been found to contain an antimicrobial sequence near its N-terminus which appears to function by a mechanism distinct from iron chelation (Tomita et al 1994). Antimicrobial peptides representing this bactericidal domain of lactoferrin were isolated following pepsin cleavage of human lactoferrin and bovine lactoferrin. The resultant antimicrobial peptide was termed lactoferricin, and has been shown to have potent broad spectrum, lethal antimicrobial properties, causing a rapid loss of colony-forming capability (Tomita et al. 1994). The antimicrobial activity of lactoferrin is often linked with lysozyme (Ellison et al. 1991). In humans both these enzymes are found in polymorphonuclear leukocytes (PMN), i.e., neutrophils, and are also present in high concentration in human mucosal secretions (Masson et al. 1966; Bullen et al. 1979; Ellison et al. 1991).

Lysozyme is a basic, cationic protein, and of particular interest because its main biological function is protection of the host against bacterial infections (Imoto 1972). It induces bacterial cell lysis by hydrolyzing the β -1-4 linked glycosidic bonds of the peptidoglycan cell wall (Imoto 1972), and is produced mainly by monocytes and

granulocytic blood cells in mammals (Jolles et al. 1966; Pruzanski et al. 1969). There are two main types of lysozyme that have been described from birds (Jolles et al. 1984) and have been termed c-type (e.g., hen egg white lysozyme) and g-type (e.g., goose egg white lysozyme). These two main types of lysozyme differ slightly in their amino acid sequence and molecular weight. The G-Type lysozyme has low cysteine and tryptophan content and has a higher molecular weight of 21 kDa than the c-type lysozyme (14 kDa) (Jolles et al. 1984). Other lysozymes have been discovered, and they differ in primary structure, physicochemical and immunological criteria (Jolles 1969; Jolles et al. 1984; Jolles 1996). These other types of lysozyme have been found in bacteria, bacteriophages, plants, the invertebrate sea star, *Asterias rubens*, and some teleosts (Tsugita et al. 1968; Jolles et al. 1975; Fange 1976). There has been lysozyme-like activity detected in an elasmobranch, using nurse shark serum, however, this activity was not studied in detail (Hedayati 1992). Lysozymes differ in amino acid sequence, molecular weight and enzymatic properties. However, amino acid sequence homology remains in the specificity of the active site responsible for the hydrolysis of the β -1-4 linkage between N-acetylmuramic acid and N-acetylglucosamine (Jolles et al. 1975).

Lysozyme has also been shown to inhibit chemotaxis of activated leukocytes (Ogundele 1998). Another anti-inflammatory function includes the inhibition of mitogen-induced lymphoblastogenesis (Gordon et al. 1979; Ogundele 1998). Lysozyme has been found to have an indirect effect on the complement system by inhibiting the PMN response towards complement-derived chemotaxins such as

anaphylatoxins C5a, C3a and C4a (Ogundele 1998). It has been reported to inhibit the classical pathway of serum complement activity in a reaction that is dose-dependent (Ogundele 1998). Its presence as an anti-inflammatory secretory component serves to protect the tissues against secondary damage during acute overwhelming infections (Ogundele 1998). In such situations excessive inflammatory reactions of the complement system would be undesirable and potentially damaging to host cells. Another role suggested for lysozyme is the immune surveillance of membranes and the possibility that it may mediate the antitumor functions of macrophages (Osserman et al. 1973; Osserman 1976).

Antimicrobial Peptides

There are several classifications for antimicrobials on the basis of their physicochemical characteristics (reviewed in Boman 1995, 2003). Those over 10 kDa are generally classified as antibacterial/antimicrobial proteins while those that are smaller are called peptides. Cathelicidins are members of a large family of peptides, all of whom share a conserved N-terminal precursor structure, cathelin, and highly heterogenous C-terminal peptides. This family includes human LL37, Bac5 and Bac7, porcine cathelicidins, and protegrins (Zanetti et al. 1995; Bucki et al. 2010). Some cathelicidins undergo extracellular proteolytic cleavage that frees the active C-terminal peptide from the precursor, while other members of the family appear active in their original non-cleaved form (Zanetti et al. 1995). Found in most mammals, LL-37 is the only member of the cathelicidin family that is expressed in humans and has shown a broad spectrum of activity against both gram-positive and gram-negative bacteria, as

well as various viruses and fungi (Bals et al. 2003; Bucki et al. 2010). It is produced mainly by leukocytes, epithelial cells and mucosal cells where it is stored in specific granules (Scott et al. 2002; Steinstraesser et al. 2009). In humans, LL-37, along with other cationic peptides, prevent infection at a whole host of body sites, such as in pulmonary and digestive systems, salivary glands, skin and ocular surfaces, as well as others (Bucki et al. 2010). LL37 assumes an amphiphatic α -helical conformation that in its original state in neutrophils is 17 kDa in size. Once processed to the mature peptide it is 5 kDa in size, which is why cathelicidins may be categorized as an antimicrobial protein, as well as a peptide (Boman 1995; Zanetti et al. 1995).

Defensins are a family of closely related, cationic antimicrobial peptides comprised of 29-45 amino acid residues. Six conserved cysteine residues are a characteristic feature of defensins that form three intramolecular disulfide bridges between the amino and carboxyl terminal regions of the peptide. This creates a cyclic, triple-stranded, amphiphatic β -sheet structure. These three disulfide bridges serve to stabilize its β -sheet structure and increase its resistance to proteolysis (Campopiano et al. 2004; Diamond et al. 2009). Defensins can be divided into α - and β -defensins, which are very similar in their three dimensional (3D) structure and in function. The differences between these two groups are (1) the fact that the β -defensins tend to be slightly larger, and some may have modified termini, and (2) linkage of the cysteine residues (Boman 2003). The α -defensins are produced constitutively and do not seem to require induction, however, most β -defensins that have been described are inducible (Diamond et al. 1991; Boman 2003). The α -defensins, which have been

studied in humans (Selsted et al. 1985), monkeys (Selsted 2004) and rodents (Ayabe et al. 2000; Ayabe et al. 2004), are found in abundance in neutrophils, macrophages and paneth cells of the small intestine (Ouellette et al. 1996; Ganz et al. 1998). They seem to have evolved to work inside the phagosome, where their propeptides are processed in the granule. These defensins are sequestered in the granules as their natural cytotoxicity could hurt the host. The β -defensins have signal peptides but no propeptides and they seem to be produced by the epithelial cells, protecting the mucosa both in the lungs and along the digestive tract (Bevins et al. 1999).

Another group that belongs to the defensin family is the θ -defensin, which are small, circular molecules, the product of two separate genes; each produces two separate defensin molecules which are linked covalently (Tang et al. 1999). The θ -defensins differ from the α - and β -defensins in that they have a double-stranded β -sheet and have only been found in primates (Tang et al. 1999; Boman 2003).

Magainins belong to a large family of amphibian amphiphatic α -helical antimicrobial peptides. They are usually 23 amino acid residues long and were originally isolated from the African clawed tree frog, *Xenopus laevis*, following observations of their continued good health after being surgically altered and left to recover under non-sterile conditions (Zasloff 1987). Magainins have been shown to have a broad spectrum of activity against gram-negative and gram-positive bacteria and fungi, and are known to reduce inflammation and promote wound healing (Zasloff 1987; Zairi et al. 2009). They also possess a potent spermicidal activity against human sperm, as well as being very effective against sexually transmitted pathogens, making

them prime candidates for exploration as spermicides (Zasloff et al. 1988; Reddy et al. 1996).

Antimicrobial peptides are also found in multiple invertebrate animals such as the penaeid shrimp (Destoumieux et al. 1997; Tassanakajon et al. 2010) and horseshoe crab (Schnapp et al. 1996). In the penaeid shrimp there are three cationic peptide families, the penaeidins (Destoumieux et al. 1997), crustins (Relf et al. 1999), and anti-lipopolysaccharide factors (ALFs). These peptides are comprised of many different isoforms and have shown activity against a variety of bacteria and fungi (reviewed in Tassanakajon et al. 2010). They are synthesized primarily in the hemocytes and are released in response to infection (Munoz et al. 2002).

Not to be forgotten, plants also produce their own antimicrobial peptides in a wide range of tissues, and express significant antimicrobial potency (Garcia-Olmedo et al. 1998). Thionin, the first plant peptide reported to have activity against plant pathogens, has been shown to alter pathogen cell membrane permeability (Fernandez de Caleyra et al. 1972; Castagnaro et al. 1992). Several families of cysteine-rich peptides have been characterized, including defensins, lipid transfer proteins (LTPs), hevein-type peptides and knottin-type peptides (reviewed in Kim et al. 2009), from plants.

Characterization of Antimicrobial Peptides

Boman (1998, 2003) has classified antimicrobial peptides, small amphiphatic peptides usually between 15 and 45 amino acid residues, on the basis of their physicochemical characteristics (Boman 1998, 2003). They are further divided into five subcategories on the basis of their amino acid composition and primary structure:

(1) anionic peptides, (2) linear amphiphatic α -helical peptides, (3) cationic peptides enriched for specific amino acids, (4) peptide fragments, and (5) peptides with cysteines that form intramolecular bonds (Boman 2000; Brogden 2005; Diamond et al. 2009). Details of the five subcategories are as follows:

- 1) Anionic peptides. Anionic peptides are present in surfactant extracts, bronchoalveolar lavage fluid, and airway epithelial cells. They require zinc as a cofactor for activity and are active against both gram-positive and gram-negative bacteria. Examples include dermcidin in humans and maximin H5 from amphibians (Brogden 2005).
- 2) Linear amphiphatic α -helical peptides. This subgroup is the most diverse and contains about 290 linear cationic α -helical peptides that tend to be less than 40 amino acid residues in length and possess a three dimensional structure with a kink or hinge in the middle. These molecules adopt an α -helical secondary structure when in contact with membranes, but are disordered in aqueous solution (Diamond et al. 2009). Examples of these peptides include the cecropins (Steiner et al. 1981), magainin (Zasloff 1987), pleurocidin from winter flounder (Cole et al. 1997) and melittin from bee venom (Fennell et al. 1968).
- 3) Cationic peptides enriched for specific amino acids. This group contains approximately 44 peptides, all of which are linear in shape and are enriched in specific amino acids. They lack cysteines residues, which makes them very flexible and fluid in solution. Examples include the bactenecins and proline-arginine-rich peptide (PR-39), both of which are rich in proline and arginine;

proline-rich abaecin from bees, and indolicidin from cattle, which is rich in tryptophan (Brogden 2005).

- 4) Peptide fragments. This fourth group is comprised of charged peptides that are fragments of larger proteins. For example, lactoferricin (mentioned earlier) is a peptide derived from the digested N-terminal portion of lactoferrin (Bellamy et al. 1993). Similarly, cathelicidins (also mentioned earlier) are peptides which are found at the C-terminus of precursors whose N-termini share a homology with the porcine serine protease known as cathelin (Zanetti et al. 1995; Diamond et al. 2009). Also in this group are peptides derived from oxygen-binding proteins, such as hemacyanin and hemoglobin derived peptides (Vizioli et al. 2002).
- 5) Peptides with intramolecular cysteine bonds. This final group is composed of roughly 380 members that all contain conserved 6-cysteine residue motifs forming intramolecular disulfide bonds and β -sheets (Brogden 2005). This defensin group is an extremely diverse group which may have originated in prokaryotes (Zhu 2007). This group has diverged into plant defensins, arthropod defensins and the β -defensins found in birds, reptiles and mammals (Wong et al. 2007; Diamond et al. 2009; Wong et al. 2009). It includes the θ -defensins, which seem to have mutated from the α -defensins, which in turn originally diverged from the β -defensins (Selsted 2004). Similar to the defensins is a liver-specific peptide, hepcidin, which is also cysteine rich, with two β -

sheets. Hepcidin exhibits both antimicrobial activity and iron-regulatory activity (Ganz 2006).

More recently, additional categories have been informally added to include newly discovered proteins and proteins with previously defined functions to which antimicrobial activity has been newly ascribed. One group is the aromatic dipeptides, which comprise low molecular weight compounds isolated from dipteran larvae (e.g. N- β -alanyl-5-S-glutathionyl-3,4-dihydroxyphenylalanine and *p*-hydroxycinnamaldehyde) (Vizioli et al. 2002).

Mechanism of Action of Antimicrobial Peptides

Most antimicrobials are cationic peptides, whose positive charge provides some selectivity towards negatively charged microbial cell outer membranes and cytoplasmic membranes. This specificity allows for differentiation between the microbial cells and host cells, and reduces cytotoxicity to host cells because their cell membrane invariably contains a higher percentage of cholesterol than bacterial cells (Diamond et al. 2009). The precise mechanism of action of antimicrobial peptides has been well studied, but still not completely understood. Their cationic charge leads to a high degree of potency in their antimicrobial activities. This is because of the presence of multiple lysine and arginine residues, a large portion of hydrophobic residues (50% or higher) and amphipathicity (Zaslhoff 1987; Hill et al. 1991; Park et al. 2000). Methods such as fluorescent dye release in model membrane systems (Ladokhin et al. 1997), ion channel formation (Christensen et al. 1988) and measurement of secondary

structure (Wu et al. 1995; Bechinger et al. 2004; Lee et al. 2004) have helped to discern several proposed mechanisms of activity.

It is generally accepted that antimicrobial peptide-mediated killing of microbes occurs through membrane permeation, but not exclusively. For example, non-membrane disruptive methods have also been observed (Diamond et al. 2009). The ability to kill bacteria via pore formation requires three key steps: binding to the bacterial membrane, aggregation within the membrane, and formation of channels. The channel formation leads to cell death via leakage of internal cell contents. In order to carry out this process, an antimicrobial peptide (often referred to as AMP) must cross the negatively charged outer membrane of gram-negative bacteria which contains lipopolysaccharides (LPS), or the outer wall of gram-positive bacteria, which contains acid polysaccharides (Hancock et al. 1997; Palfy et al. 2009). The three most established pore forming models are the barrel-stave pore, the toroidal pore, and the carpet model (Lehrer et al. 1989; Hancock 1997; Oren et al. 1998; Palfy et al. 2009). In the barrel-stave pore model, AMP binds to the negatively charged membrane to form dimers or multimers. Multimers of AMP cross the cell membrane so that the hydrophobic region is facing the lipid bilayer, and the hydrophilic region is facing the lumen of the pore. The assembled peptides form barrel-like channels resembling staves (Ben-Efraim et al. 1997). The toroidal pore model shares a common mechanism with the barrel-stave pore model. However, in this model, the AMP forms a monolayer by connecting the outer and inner lipid layers in the pore (Lehrer 1989; Hancock 1997; Oren et al. 1998; Palfy et al. 2009). The carpet model has peptides

covering the outer surface of the membrane, thus the peptides act as detergents, disrupting the membrane bilayer after reaching a threshold concentration. The pores are formed from micelle-like units (Oren et al. 1998; Palffy et al. 2009).

Additional models have been suggested that are specific to one or other AMP. This includes molecular electroporation, where some peptides are able to create electrostatic potentials across the bilayer sufficient for pore generation (Chan et al. 2006; Palffy et al. 2009). Other methods involve peptides binding to the endotoxin moieties of bacterial LPS (such as defensins and cathelicidins) (Bals 2000; Palffy et al. 2009). Some AMPs inhibit DNA synthesis, protein synthesis, or both (Gennaro et al. 2002; Boman 2003). Histatin targets the mitochondria of fungal pathogens (Tsai et al. 1998).

Histones historically have been associated with DNA stabilization, playing a crucial role in the packaging of DNA, thereby ensuring efficient replication and segregation of the chromosomes. Histone gene transcription is coordinated with the cell cycle and regulated by cyclins and D-type cyclins, CDKs (Ewen 2000). They are divided into two functional types: 1) core histones, which are comprised of histones H2A, H2B, H3 and H4, and together form an octameric complex (about 146 basepairs of DNA) to create the nucleosome; and 2) linker histones, H1, which seal loops of DNA that enter and leave nucleosomes and condense these structures into compact, higher order fibers. The structure of core histones does not differ much among species. The H1 histones, however, are the largest and most heterogeneous family of histones and much more varied (Smith 1991; Hiemstra et al. 1993). These proteins show structural variations

not only among but also within species (Kawasaki et al. 2008). These variations can be explained by any number of post-translational modifications such as acetylation, methylation, poly-(ADP ribosyl)ation, ubiquitination, sumoylation and perhaps even phosphorylation (Parseghian et al. 2006).

Despite being historically associated with DNA packaging, histones are not confined to the nucleus. It has been found that after biosynthesis in the cytosol by free ribosomes, a limited amount of histone is accumulated. These are imported to the nucleus via karyopherins (also known as importins), while the excess (unacetylated) histones accumulate in the cytoplasm, especially in rapidly regenerating or transcriptionally active cells (Zlatanova et al. 1990; Watson et al. 1995; Chang et al. 1997; Augusto LA 2003). Secretion of cytoplasmic histone extracellularly has been observed (Watabe et al. 1996; Brix et al. 1998; Augusto LA 2003), and extracellular histones are released from apoptotic cells (Schwartz et al. 1993). Histones may also be found on the cell surface, as evidenced by binding of sulfated polysaccharide D2S to the surface of T-cells. Using ligand-studies and immunoblotting techniques, histone H2 was found to be the ligand on the surface of the T-cell (Watson et al. 1995). Consequently, histones are quite ubiquitous, having been shown to be present in the cell nucleus, cytosol, cell membrane, and extracellular environment (Parseghian et al. 2006).

There are several possible explanations as to why histones, usually associated with DNA stabilization, have shown antimicrobial activity. It has been suggested that a structural motif of histone, which strongly binds to DNA, may be used as an

antimicrobial peptide to bind to the cell membrane of invading microorganisms (Park et al. 1996). Studies (Frank et al. 1990; Lehrer et al. 1992) have shown that one main biological action of antimicrobial peptides such as defensins and bactenecins involve lipid and membrane binding. Since a single biologically active compound can have more than one kind of macromolecule-binding activities, it can be hypothesized that the isolated peptides adopted the histone-like structure to bind strongly to lipids of cell membranes, which is necessary for antimicrobial activity (Frank et al. 1990; Lehrer et al. 1992; Park et al. 1996).

Other studies have revealed yet another function of histones. Researchers have observed that histones have a remarkably high affinity for LPS. In lung epithelial cell lines they found that the most efficient LPS binding proteins were two histones, H2A.1 and H4. However, to be effective, the entire 3D structure of the histone is required to be intact (Augusto et al, 2003). It has also been reported that the major LPS-binding protein in the brain is an isoform of histone H1, predominantly located at the neuronal cell surface (Bolton et al. 1997).

Histones as Novel Antimicrobial Peptides

In leukocytes, antimicrobial peptides are sequestered in granules. In response to an infection, the leukocyte is activated and, following phagocytosis of microorganisms, its cell content degranulates releasing hydrolyzing enzymes, antimicrobial peptides and other effector molecules to the infection site. As these granules contain acid soluble enzymes such as lysozyme, ribonucleases and deoxyribonucleases; the nature of antimicrobial substances was difficult to distinguish from the histones and

protamines found in the nuclei (Zeya et al. 1966; Isenberg 1979). Investigators have since been able to attribute antimicrobial activity specifically to histones and their related fragments, working independently or synergistically with each other and other peptides (Rose et al. 1998; Agerberth et al. 2000; Kim et al. 2000; Patrzykat et al. 2001; Kawasaki et al. 2008). Histones that possess such broad spectrum antimicrobial activity have been found in shrimp (Patat et al. 2004), teleost fish (Robinette et al. 1998; Noga et al. 2001; Richards et al. 2001; Fernandes et al. 2002; Bergsson et al. 2005), frog (Kawasaki et al. 2003), chicken (Silphaduang et al. 2006; Li et al. 2007) and mammals (Hiemstra et al. 1993; Rose et al. 1998; Kim et al. 2002; Howell et al. 2003; Jacobsen et al. 2005) (reviewed in Kawasaki and Iwamuro 2008).

Purified histone H1 was found to be expressed in the cytoplasm of intestinal villus epithelial cells, and upon detachment of the epithelial cell, was released as the cell underwent programmed cell death (i.e., apoptosis) (Rose et al. 1998). It was suggested that H1 may protect villus epithelial cells against penetration by microorganisms (Rose et al. 1998). Immunohistochemistry studies show that H1 is found in the nucleus, affording the cell continued protection even after the microorganism had penetrated through the cell membrane (Rose et al. 1998). In flounder, there is additional evidence that synthesized histone H1 may work synergistically with flounder pleurocidin to potentiate and enhance its antibiotic effects against fish pathogens (Patrzykat et al. 2001). In the chicken, full length histone H1, as well as H2B, were purified from extracts of the ovary and oviduct, and were active against both gram negative and gram positive bacteria, but a high salt

concentration was required for histone H1 to express the activity against the gram negative bacteria (Silphaduang et al. 2006). A histone H1 fragment has been isolated from skin secretions of rainbow trout, termed oncorhyncin II, with broad spectrum antimicrobial activity against not only bacteria, but also fungi (Fernandes et al. 2004).

Histone H2A is the best studied member of this family of proteins, and the full length protein has been isolated from human placenta (Kim et al. 2002), chicken liver (Li et al. 2007), and rainbow trout (Fernandes et al. 2002). Histone H2A fragment was isolated from gastric tissue of the Asian toad and results of the study provided further evidence of the association of histones with cells undergoing apoptosis (Park et al. 1996). The toad histone H2A fragment was found to be a precursor of Buforin I, a potent 39 amino acid antimicrobial peptide. These instances provided further evidence that histones play a major role in host defense (Park et al. 1996, 2000). By substituting an amino acid on Buforin I, a H2A-derived antimicrobial peptide was generated that showed a broad-spectrum of activity against several microorganisms, but did not kill microbes by cell lysis. This modified antimicrobial agent had a strong affinity for DNA and RNA, suggesting that it targeted intracellular substances like nucleic acids (Park et al. 2000). This observation indicates an enormous potential for the production of a new class of antibiotics (Barra et al. 1998; Boman 1998). Two other forms of histone H2A-related peptides (fragments) have been found in the skin mucus of fish, Parasin I, from the skin mucus of a wounded catfish (Park et al. 1998) and hipposin, from the Atlantic halibut (Birkemo et al. 2003).

Histone H2B was first isolated from the cytoplasm of mouse (murine) macrophage cell lines, RAW264.7 and J774A.1 (Hiemstra et al. 1993). It has also been isolated from colonic epithelial cells in tandem with H1 (Howell et al. 2003). Calf intestinal H2B was found to have anti-parasitic activity, similar to the histone H1-like protein isolated from the skin, gill, and spleen extracts of rainbow trout and sunshine bass (Noga et al. 2001). Histone H2B fragments have been isolated from extracts of human blister wound fluid, which displayed very high activity against gram positive bacteria (Frohm et al. 1996).

There are few reports of the antimicrobial activity of histones H3 and H4, not because they are less studied, but because of their pattern of co-purifying with other histones like histone H2B (Kawasaki et al. 2008). Recently, however, investigators have reported on the antimicrobial activity of histone H4 (Lee et al. 2009). A novel histone antimicrobial peptide, synthesized from buforin II, shows homology to histone H3 with broad spectrum antimicrobial activity (Tsao et al. 2009).

Augusto et al. (2003) observed that the affinity of histones for bacterial lipopolysaccharides (LPS) is higher than that of polymyxin B, an antibiotic known to block several biological activities of LPS. This affinity indicates that the therapeutic potential of histone analogues may (as with other LPS-binding molecules) reduce bacterial growth, facilitate bacterial opsonization, or directly block the interaction of LPS with host pro-inflammatory pathways.

Immunomodulatory Effects of Antimicrobial Peptides

The synthesis of enteric defensins in human fetal tissue starts 13.5-17 weeks after gestation, and it has been shown that LL37 and members of the defensin family are present in amniotic fluid (Mallow et al. 1996; Yoshio et al. 2004). It was once the prevailing thought that an antimicrobial peptide's only function was as a microbicidal agent. It has since come to light that this is only one aspect of these astonishing molecules. Antimicrobial peptides (AMPs) have the capacity to regulate epithelial cell proliferation, enhance wound healing (Frohm et al. 1996), inhibit or induce pro-inflammatory cytokines (Nagaoka et al. 2001), take part in angiogenic and anti-angiogenic processes (Li et al. 2000), stimulate chemokine production (Bowdish et al. 2005), facilitate chemotaxis of various leukocytes (Huang et al. 1997), influence mast cell degranulation (Befus et al. 1999) and modulate host cell gene expression (Kelly et al. 2006). To the surprise of mainstream immunologists, these relatively obscure peptides have been implicated in helping to modulate adaptive immune function. A number of studies have reported that co-administering antimicrobial peptides with relatively benign antigens results in the enhancement of the host's cell mediated and humoral immune responses to these antigens (Lillard et al. 1999; Tani et al. 2000; Brogden et al. 2003). These findings strongly implicate α -defensins as immune adjuvants that promote T cell-dependent cellular immunity as well as antigen-specific immunoglobulin production (Lillard et al. 1999). Furthermore, it has been postulated that antimicrobial peptides may be modulating lymphocyte responses, modifying cytokine expression during antigen presentation and possibly causing maturation of immature dendritic cells

by inducing co-stimulatory molecules, resulting in more effective antigen presentation and subsequent robust T cell activation (Funderburg et al. 2007; Diamond et al. 2009).

Peptides have also been shown to act as cofactors to lower lethality of a hyperinflammatory response (Steinstraesser et al. 2009). Endotoxin, found on the outer layer of gram-negative bacteria, is recognized as harmful and activates the innate immune system. In some cases, antibiotics can cause endotoxin release which can be neutralized by the antimicrobial peptide LL-37, thus inhibiting the production of pro-inflammatory cytokines such as tumor necrosis factor- α and IL-6 (Zanetti et al. 1995; Scott et al. 2002).

Significance of Antimicrobial Peptides

The number of known antimicrobial peptides is rapidly increasing. So far over 1,220 peptides have been characterized, including over 940 peptides from eukaryotic organisms (Steinstraesser et al. 2009). There are three databases that list antimicrobial peptides. These are the ANTIMIC, AMPer and APD2 (Brahmachary et al. 2004; Fjell et al. 2007; Wang et al. 2009) databases. AMPs differ in their structure, physicochemical characteristics and biological function. They also differ in their mechanisms of action, as well as their antimicrobial activities (non-cationic peptides tend to have weaker bactericidal activity than do cationic peptides) (Vizioli et al. 2002). It has been shown that antimicrobial peptides can kill susceptible bacteria *in vitro* at concentrations up to 4 $\mu\text{g}/\text{ml}$ (Hancock et al. 1998); beyond that concentration there is the risk of host cell toxicity. Antimicrobial peptides have been shown to play a

significant role in the prevention of disease (Hancock et al. 1998; Rivas-Santiago et al. 2009). There are scores of studies on the susceptibility to infectious disease on the basis of the level of antimicrobial peptide production by the host. During primary infection, the level of inflammation is directly related to the immune response mounted by the host. Recruitment of inflammatory cells by chemokines and production of pro-inflammatory cytokines and release of antimicrobial peptides will determine the outcome of infection (Rivas-Santiago et al. 2009). Antimicrobial peptides play key roles in infectious diseases of the skin. In atopic dermatitis, viral and bacterial infections complicate successful therapy as they can lead to cutaneous inflammation. It has been shown that these patients have a significantly lower production of dermcidin 1 than do healthy subjects (Schitteck et al. 2001; Rivas-Santiago et al. 2009). Another skin condition, atopic eczema, is associated with decreased expression of human β -defensin-2 (HBD-2), HBD-3 and LL-37, which can lead to skin infections with *Staphylococcus aureus* (Ong et al. 2002; Nomura et al. 2003; Rivas-Santiago et al. 2009). Psoriasis is another example of human inflammatory skin disease associated with abnormal antimicrobial peptide expression. Cathelicidin is increased in lesional skin in these patients (Ong et al. 2002; Rivas-Santiago et al. 2009). These studies have important implications on the use of cathelicidins and others in skin infection control. Clinical applications of antimicrobial peptides in treating burn wounds have also been examined. In one study, transient cutaneous adenoviral delivery of LL-37 resulted in significant bacterial inhibition that might be a potential

adjunct for wound treatment in the near future (Jacobsen et al. 2005; Rivas-Santiago et al. 2009).

Antimicrobial peptides have also been studied in gastrointestinal infections as the healthy intestinal tract is characterized by a sensitive balance of host antimicrobial peptides and intestinal microbes. For example in some African adults, low α -defensin expression appears to be associated with a higher risk of infectious diarrhea (Kelly et al. 2006; Rivas-Santiago et al. 2009).

Members of the three main peptide families are found in the oral cavity: LL-37, α - and β -defensins and peptides with an unusually high proportion of specific amino acids, such as histatins (Dale et al. 2006; Rivas-Santiago et al. 2009). In Morbus Kostmann, a genetic disorder, patients experience frequent oral bacterial infections and severe periodontal disease, which correlate with a deficiency in the production of cathelicidin peptide LL-37 and α - and β -defensins (Dale et al. 2006; Rivas-Santiago et al. 2009).

Systemic infectious diseases that are caused by viruses such as the HIV virus have been shown to be vulnerable to β -defensins as they seem to compete for the same chemokine receptor, so the virus is unable to enter the cell. The β -defensin then seems to confer a protective response to the host (Garzino-Demo 2007; Rivas-Santiago et al. 2009).

Lung infections caused by infiltration and growth of *Mycobacterium tuberculosis* have been shown to be marginally controlled by the presence of high levels of

cathelicidin, LL-37, in epithelial cells, neutrophils, and alveolar macrophages (Rivas-Santiago et al. 2006).

The studies mentioned above all suggest there is efficacy in the possible use of certain antimicrobial peptides in therapy as an alternative treatment option in the management of infectious diseases (Rivas-Santiago et al. 2009).

Significance of Elasmobranchi as a Model System

In the rapidly evolving area of inquiry into the nature of antimicrobial peptides, there are many questions that still exist. These include:

- How ubiquitous are these proteins and peptides in nature?
- What other animals express these proteins and peptides?
- What are their mechanisms of action?
- How have these proteins and peptides evolved in the development of vertebrate and invertebrate immunity?

There is a need for more study in this area, using varied animal systems. The nurse shark, *Ginglymostoma cirratum*, which belongs to the class *Elasmobranchi*, is a basal vertebrate with a fully developed innate and adaptive immune system. This basal animal is strategically placed in the phylogenetic lineage and is a good model to study unresolved questions regarding antimicrobial peptides in primitive vertebrates. The nurse shark's natural antibodies, spontaneous cytotoxicity, and complement have been studied, and are believed to work in concert to form an immunosurveillance system seeking out new antigens (Rudikoff et al. 1970; Clem et al. 1982; Pettey et al.

1983; Smith et al. 1986; Flajnik 1994; Parham 1995). In addition, innate system components such as antibacterial proteins/peptides and other antimicrobial substances may form the basis of innate immunity necessary to maintain a healthy animal, particularly during times of stress. Nurse sharks inhabit tropical and subtropical waters on the continental shelf, and can be found along the Western and Eastern Atlantic coasts; along the islands of the Caribbean; as well as the Eastern Pacific coast. Nurse sharks have been known to inhabit polluted waters, as they are bottom dwellers of shallow warm waters (Fange et al. 1981). In this environment the nurse shark is at a greater risk to infectious agents than animals that inhabit deeper and more open waters (which are less likely to be polluted), and runs a greater risk of physical trauma as a result of man's intrusion into its habitat (Reif 1978). For these reasons, an efficient immune system with innate and adaptive immune mechanisms is essential for its survival. Because of the critical role of innate immunity in the development and survival of this species, *Ginglymostoma cirratum* makes a good animal model to study, aided by the fact that it is one of a few shark species that can be successfully maintained in captivity for long periods (Clark 1963). The nurse shark has a less well developed adaptive immune system compared to mammals, therefore it would be expected that a non-specific antibacterial response to pathogens would play a more significant role when mounting an immediate immune response.

Goals and Hypothesis

This study was undertaken to test the hypothesis that in addition to lysozyme, nurse shark peripheral blood leukocytes possess antibacterial factors that include histone

fragments. The specific goal was to demonstrate antibacterial activity in crude leukocyte lysates, against a target bacterium, *Planococcus citreus*, and to determine its mode of action.

III. MATERIALS AND METHODS

A. MATERIALS

Details of the buffers, reagents, solutions, culture media preparation, protocols for the preparation of acrylamide gels, western blot buffers and antisera preparation and concentration can be found in Appendix I.

B. METHODS

Blood Collection

Initially several (8-10) nurse sharks (*Ginglymostoma cirratum*), males and females, ranging in size from 1.5 -2 meters (5-7 feet) were maintained in a seawater channel at the Keys Marine Laboratory, Long Key, Florida Keys. Following the loss of all animals in a hurricane, a newly acquired, single female animal, about 1.2 meters (4 ½ feet), was maintained and bled at 6-8 week intervals to obtain 60 ml of blood. The shark was anesthetized in a rectangular tank of seawater containing 1 ppm 3-aminobenzoic acid ethyl ester (methanesulfonate salt). The anesthetized shark was bled by placing it in a horizontal position on its back, on a clean surface, and withdrawing blood with a 20 gauge, 1½ inch needle from the caudal vein using a 60 ml syringe. The animal was then returned to the channel and manually moved back and forth through the water until normal gill respiration returned before release. This work was conducted with institutional IACUC approval.

Preparation of Cytospins

Blood smears were prepared from a nurse shark leukocyte-enriched suspension to evaluate cell morphology and to acquire differential counts. Nurse shark heparinized whole blood (1000 units/ml) was left to settle for 2 hours. The buffy coat was removed (with a thin layer of erythrocytes attached) and spun at 435 x/g (1240 rpm) for 10 minutes (min) at 15°C. The leukocyte enriched pellet was resuspended and washed with sRPMI at 435 x/g (1240 rpm) for 10 min at 15°C. The wash was decanted and the pellet was resuspended to 1×10^5 cells/ml. 250 μ l of the cell suspension was loaded onto a cytospin chamber loaded with a poly-L-Lysine coated microscope slide and centrifuged in a cytocentrifuge (Cytopro, *Wescor*), at 250 x/g (1200 rpm) for 3 min at room temperature. The leukocyte enriched monolayer was air-dried and stained with leukostat stain for morphological identification of nurse shark peripheral blood cell populations.

Isolation of Peripheral Blood Leukocytes

Freshly collected nurse shark blood was aliquoted in 15 ml polypropylene, screw cap tubes containing anticoagulant. Blood (9 ml) was added to 1 ml of 3.8% trisodium citrate in shark RPMI media (0.35M urea, 0.2 M NaCl, pH 7.4), to give a final concentration of 0.38% anticoagulant. The tube was inverted gently several times until complete mixing was achieved, and blood samples were transported on ice to the lab for processing.

Anticoagulated blood was diluted with an equal volume of shark RPMI. Using a long pasteur pipette, 24 ml of the diluted blood sample was withdrawn and layered carefully onto the surface of 18 ml of a gradient mixture consisting of two parts Lymphocyte Separation Media, LSM (*Sigma, MO*), and one part physiological saline (0.15 M NaCl) in a 50 ml centrifuge tube. The sample was centrifuged at 435 x/g (1240 rpm) for 30 min at 15°C in a Beckman GPR centrifuge (brake off). A small volume of whole blood was retained to determine the total white cell count and differential counts. Following centrifugation, the cloudy interface layer along with the thin buffy coat layer present on the surface of sedimented erythrocytes was removed and transferred to a sterile 15 ml tube. The harvested leukocytes were separated by centrifugation at 435 x/g (1240 rpm) for 20 min at 15°C. Any remaining contaminating erythrocytes in the pellet were lysed by hypotonic lysis; the pellet was resuspended in approximately 5 times its volume in 0.2% NaCl by inverting several times to resuspend cells completely (this permitted all cells to be lysed by the hypotonic lysing solution). Then an equal volume of 2.5% NaCl was added to the solution of 0.2% NaCl solution to neutralize the effect of the lysing solution. The suspension was centrifuged at 435 x/g (1240 rpm) for 15 min at 10°C and the supernatant discarded. The pellet of isolated leukocytes was resuspended in 1-2 ml of 0.25 M NaCl, isotonic for shark leukocytes. Using a small aliquot of the leukocyte suspension, a total cell count was determined manually using a haemocytometer and used to calculate the leukocyte yield. A viability test was also performed (employing the trypan blue exclusion test) to confirm

that cells were viable and intact and not 'leaking' intracellular contents. The cell suspension was stored at -20°C until ready to use.

Trypan Blue Exclusion Test

The trypan blue exclusion test was used to determine the viability of shark cells because live, viable cells with intact cell membranes, exclude certain dyes, such as trypan blue. For experimental purposes a cell viability of 95% or higher was deemed optimal for biological assays. A 10 µl aliquot of cell suspension was diluted in 1 ml 0.25M NaCl and 10 µl removed and mixed 1:2 with cell viability reagent. After 3 min incubation at room temperature (RT), a drop of suspension was applied to a haemocytometer and viewed under the 40x objective lens. The stained (nonviable) and unstained (viable) cells were counted separately up to a total of 200 cells. The percent viability was calculated as follows:

$$\text{Percent viable cells} = \frac{\text{\# viable cells per 200 cells}}{200 \text{ cells}} \times 100$$

Preparation of Leukocyte Lysates

Cell lysates were prepared from the frozen suspended pellets (isolated using LSM) by first freezing and thawing to facilitate lysis. The cell pellets were thawed at room temperature, refrozen at -80°C, and then thawed again. Once thawed, the cell suspension was held on ice. The repeated freeze-thaw treatment facilitated subsequent disruption of cells by mechanical shearing using a glass homogenizer and/or ultrasonic disruption in a sonicator (Sonicator 3000, *Misonix*). For sonication of

cell pellet, 0.25M NaCl was added to the cell suspension. A 2 ml cell suspension was placed in a 15 ml screw cap tube, and immersed in a beaker filled with ice and sonicated at 18-27 watts for 10 min with 1 min pulses and 2 min rest periods in between pulses. Efficiency of disruption was confirmed by microscopic examination of the sonicated suspension for complete disruption of cells with intracellular contents expelled. The resulting cell lysate was collected by centrifuged for 90 min at 21,000 xg at 7°C (Micromaxx FF, IEC) to pellet the debris. The cell lysate supernatant (CLS) was removed and stored at -20°C as small aliquots of 2 ml in eppendorf tubes. Protein concentration of each leukocyte lysate preparation was determined using the BCA colorimetric protein assay protocol (Pierce, IL).

Concentration and Dialysis of Lysates

Each cell lysate supernatant was thawed and clarified by centrifugation for 5 min at 1000 xg (Micromaxx FF, IEC) to sediment any precipitated material which may have formed as a result of freezing. Lyophilization was used to concentrate samples.

Dialysis: For chromatography, lysate samples were dialyzed against the column equilibration buffer using Spectra Por Dialysis Tubing with a 3000-D cutoff. Dialysis was carried out for up to 8 hours at 4°C with buffer changes every 2 hours (h).

Lyophilization: Before being subjected to lyophilization, high salt samples (e.g., column fractions eluted at high salt) were first dialyzed (as per above). The thawed samples were placed in 50 ml polypropylene tubes and freeze-dried (Freezedryer,

Labconco) for 12-18 hours. The lyophilized samples were reconstituted in a small volume of ddH₂O to obtain a 4-10 fold concentration of the sample.

Detection and Quantitation of Antibacterial Activity

Screening methods

(1) Lysoplate assay

The lysoplate assay was used as a screening method to assay for lysozyme (muramidase) activity. The lysoplate method was originally developed by Osserman and Lawlor (1966) and later modified by Manchenko (1994). A 0.12% (1.2 mg/ml) stock suspension of lyophilized *Micrococcus lysodeikticus* cell walls in 0.06M phosphate buffer, pH 6.2 was prepared and stored as 50 ml aliquots at 4°C. Lysoplates were set up by preparing an agarose lawn in 0.06M PB, pH 6.2, containing a suspension of *Micrococcus lysodeikticus* cell walls (0.06% final concentration). To prepare a lysoplate, the *Micrococcus lysodeikticus* cell wall suspension was removed from the cold and equilibrated to 55°C before being added to the agar medium.

To prepare the plates equal volumes (5 ml each) of 2% agarose and 0.12% bacterial cell wall suspension, equilibrated to 55°C for one hour in a water bath, were gently mixed together to a final concentration of 1% agarose and 0.06% cell wall suspension, and poured into a square petri dish marked with a grid (*Fisher Scientific, GA*). Wells (3 mm diameter) were punched into the solidified agar at the

center of each grid square using the sterile tip of a 3mm diameter template cutter (ICN, OH). The cell lysate samples were thawed and kept on ice prior to the assay. Each well was filled with 5 μ l of test sample. Negative controls consisted of distilled water, buffer or salt solutions, while chicken egg white lysozyme (final concentration 0.01 mg/ml) was used as a positive control. Assays were set up in duplicate. Once sample material diffused from the well into the agar, the plates were inverted and incubated at 37°C for 24 hours. Plates were examined for zones of clearing (indicative of cell wall lysis) around the wells and the diameter of the zones (measured as an average size of duplicate tests) recorded. Lysozyme activity was calculated in arbitrary units, as follows. The diameter of the well (3 mm) was subtracted from the diameter of the zone of clearing, and using the relationship of 0.1 mm diameter size equal to 1 unit (U) of enzyme activity (Lehrer et al. 1991) the enzymatic activity was estimated.

(2) Agar Diffusion assay

The agar diffusion assay was used as an initial screening method to detect antibacterial activity. The original protocol developed by Lehrer (1991) was modified. In place of marine broth an artificial seawater medium, enriched with peptone and yeast extract (Makemson et al. 1998) was used, which provided a clearer agar medium (minimal precipitation) and permitted sharper visualization of antibacterial activity. The supplemented sea water medium supported good growth of *Planococcus citreus*, used as the target bacterium in experiments.

Planococcus citreus, a salt tolerant Gram positive coccus, was used as the target organism in the antibacterial assay to detect antibacterial activity of shark cell lysates. Stock cultures of *P. citreus* were maintained at 30°C on agar plates and slants of artificial sea water complete agar (SCA) and subcultured every other day. Stock cultures were stored at 4°C. From a 48h SCA plate culture, *P. citreus* was inoculated into 50 ml artificial sea water complete broth (SCM) in a 125 ml conical flask and grown to log phase for 6-8 hours at 30°C in an incubator. A 5 ml aliquot of broth culture was then removed and added to fresh medium (50 ml) and incubated further for 8 hours at 30°C. The broth culture was centrifuged (1085 x g for 5-7 m) and the sedimented bacterial pellet was resuspended in SCM. For the radial diffusion assay, the resuspended bacterial pellet was washed twice with 3.2% NaCl solution. The bacterial suspension was standardized to an OD_{580 nm} of 0.5 which corresponded to a bacterial count of approximately 10⁶ CFU/ml. Before use in the agar diffusion assay, the agar stored in screw cap tubes was melted in a boiling waterbath (Tek-Bath, Tek-Pro), then cooled to 44°C in a water bath before addition of 100µl of the target organism *P. citreus*, (10⁶ cells/ml), to the molten agar. The mixture was stirred carefully to evenly suspend the bacteria, poured into 10 cm sterile petri dishes, and allowed to solidify at room temperature. The assay plates were stored at room temperature and used within the hour to set up the acid-urea overlay assays.

The *P. citreus* inoculum for microdilution growth assays was prepared as described above for the agar diffusion assays, however, the sedimented bacterial pellet was resuspended in SCM, and standardized to an OD_{580 nm} of 0.15 which corresponded to a bacterial concentration of approximately 2.5 x 10⁴ CFU/ml (determined as above).

Microdilution growth assay

The agar diffusion assay was limited in sensitivity and only provided a rough estimate of the level of antibacterial activity. It was necessary to design an assay with increased sensitivity to detect antibacterial activity and which could be used to measure the level of activity. Since no such assay had been described for shark antibacterial proteins/peptides, an essential part of this study was to develop a microdilution growth assay. Initially the assay was developed using sea water complete broth (SCM) medium and *Planococcus citreus* as the target organism. The reaction mix for the assay consisted of 50 µl of test sample (cell lysate supernatant), 75µl of 2x SCM and 100 µl of log phase *P. citreus* standardized to 2.5 x 10⁴ CFU/ml. The assay was set up in a polypropylene microwell plate (VWR, PA) and each sample was set up in triplicate. The plates were mixed on a microplate shaker for 5 min, and absorbance read at OD_{570nm}, which served as a 0 h reading (t=0). The plate was further incubated at 30°C for 12 hours and absorbance read again every two hours at OD_{570nm}. Extrapolating from the growth curve of *P. citreus* the end point of the assay was established at 12 h when maximum growth is obtained. Assays were set up in triplicate as follows, with each triplicate consisting of a medium control (225 µl of 2x

SCM), culture control (50 μ l of 1x SCM as diluent, 75 μ l of 2x SCM and 100 μ l of log phase *P. citreus*), lysate control (50 μ l of cell lysate supernatant and 175 μ l of 2x SCM), and defensin control (50 μ l α -defensin, HNP1 (25 μ g/ml), 75 μ l of 2x SCM and 100 μ l of log phase *P. citreus*).

To determine optimal assay conditions for maximal sensitivity for detecting antibacterial activity, the assay was set up at varying incubation temperatures, pH, and salinity, and using different cell densities of the *P. citreus* culture. Once conditions were optimized, the standard assay was routinely used to detect antibacterial activity in samples.

Optimal pH for detecting antibacterial activity was determined by setting up standard assays using assay medium adjusted to pH values of 4.0, 5.5, 6.5, 7.5 and 8.5. One volume of the sample was mixed with two volumes of the pH adjusted medium and 50 μ l was used to set up the standard assay using pH-adjusted medium corresponding to that of the treated sample. A similar protocol was followed to determine optimal ionicity using media adjusted to NaCl concentrations ranging from 0.3 M to 0.6 M. Controls as described above for the standard assay were included. For all experimental assays, the normal growth of *P. citreus* and the antibacterial effect on the growth of *P. citreus* was monitored by measuring OD at specific time intervals up to 12 hours. Optimal incubation temperature was determined by performing the assay at 30°C and RT (~25°C).

To determine the optimal concentration of *P. citreus* to develop a highly sensitive assay capable of detecting relatively low levels of antibacterial activity, assays were

designed using three different concentrations of target organism. A bacterial suspension, standardized to 0.5 at OD_{580 nm}, which corresponded to a bacterial count of approximately 10⁶ CFU/ml, was diluted 1:10 (1x10⁵ CFU/ml), 1:20 (5x10⁴ CFU/ml) and 1:40 (2.5x10⁴ CFU/ml) with 1x SCM. Five hundred µl of each concentration of bacterial suspension was plated onto SWA plates and incubated for 18 hours at 30°C. The colony forming units were calculated from these plates to confirm each bacterial suspension concentration.

The microdilution growth assay was also used as a quantitative assay to measure the level of antibacterial activity in samples. Quantitative assays were set up by adding 50 µl of the test sample to 50 µl of diluent (1x SCM) in the second well and continuing to serially dilute the sample up to 1:32 dilution using 2-fold serial dilution in SCM. The first well contained 50 µl of undiluted sample. Each assay was set up in triplicate. The microtiter plates were mixed on a microplate shaker for 5 min, and absorbance read at OD_{570nm}. The plate was further incubated at 30°C for 12 hours and absorbance read again at OD_{570nm}. The level of activity was calculated from OD reading and expressed as a titer and measured as percent inhibition using the formula:

$$\text{Percent Inhibition} = \frac{\text{OD}_{570\text{nm}} \text{ of } P. \textit{citreus} \text{ control} - \text{OD}_{570\text{nm}} \text{ of sample}}{\text{OD}_{570\text{nm}} \text{ of } Planococcus \textit{citreus} \text{ control}} \times 100$$

Antibacterial titer was defined as the inverse of the highest dilution of the test sample that showed inhibition (>5%) of growth of the target organism (Herbert et al. 1977).

Fractionation of Cell Lysates

Screening of cell lysate supernatant showed two distinctly different activities, a lysozyme-like activity and non-lysozyme-like antibacterial activity. In order to separate the molecules responsible for these two activities, ion exchange and affinity chromatography were undertaken. Earlier studies showed that recovery of antibacterial activity from cation exchange chromatography was not optimal (low recovery of activity). Therefore, initial fractionation of cell lysates was accomplished by anion exchange chromatography employing a weak (DE-52 cellulose, *Whatman*), a medium strength (DE Sepharose, *Pharmacia*), and a strong (Q-sepharose, *Pharmacia*) ion exchanger. The use of different ion exchangers was carried out to determine which protocol would achieve optimal separation and yield significant recovery of antibacterial activity. Following chromatographic separation active fractions were pooled and further fractionated by affinity chromatography on a heparin affinity column. All chromatography was carried out at room temperature (25°C) and columns were run using a peristaltic pump at a constant flow rate. Fractions were collected and OD_{280/260 nm} read manually on a spectrophotometer (Ultraspec 3000, *Pharmacia*). Fractions were assayed for antibacterial activity using the standard microdilution growth assay while lysozyme activity was screened using the lysoplate assay. Active fractions were pooled, concentrated by freeze-drying, and reconstituted in smaller volumes of ddH₂O. The concentrated pools were stored at -20°C until needed for further processing.

Anion exchange chromatography using DE 52 Cellulose

Preswollen diethyl aminoethyl cellulose (*DE 52, Whatman*), a microgranular cellulose matrix, was rehydrated in 0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5. The pH of the suspension was adjusted to 7.5 with 10 N NaOH. The buffer was decanted and discarded, and the gel washed several times with equilibration buffer (0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5) until the pH and ionicity of the gel mix was the same as the equilibration buffer. A column measuring 1.0 cm diameter x 4.5 cm in length was poured (bed volume of 3.6 ml), and equilibrated with 20 ml equilibration buffer. The flow rate was adjusted to 0.5 ml/min and 0.75 ml fractions were collected. Sample material was mixed with equilibration buffer in a ratio of 3:1, before being applied to the column. The column was washed until all unbound protein had been collected and the OD_{280nm} of the eluant was the same as the equilibration buffer. Proteins bound to the column were eluted with a linear NaCl gradient formed in a gradient mixer (*Hoefer SG-100, Pharmacia*) using an equal volume (20 ml) of elution buffer (0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5) and equilibration buffer. After the gradient, an additional 10 ml of high salt buffer (0.02 M Tris-HCl, 1 M NaCl, pH 7.5) was applied to the column to elute all tightly bound protein. Fractions were assayed for antibacterial activity and lysozyme as described above. Those exhibiting antibacterial activity were pooled and concentrated by lyophilization and stored at -20°C until needed.

Anion exchange chromatography using Q Sepharose or DE Sepharose

Q Sepharose and DE Sepharose are beaded agarose matrices (*Pharmacia, NJ*). Material was washed several times with equilibration buffer (0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5) to remove the ethanol preservative and a column measuring 1.0 cm diameter x 4.5 cm in length (bed volume of 3.8 ml) was prepared according to the procedure described above for DE cellulose and run under similar conditions except the flow rate was adjusted to 0.5 ml/min and 1 ml fractions were collected. Active fractions were pooled, dialyzed, concentrated by freeze-drying, and stored at -20°C until needed.

Heparin-sepharose affinity chromatography

Heparin is a glycosaminoglycan which serves as an effective affinity binding and ion exchange ligand for a wide range of bio-molecules and has been shown to bind enzymes/proteins that act on nucleic acids. Heparin-Sepharose, a highly cross-linked agarose matrix (*Pharmacia, NJ*), was washed several times with equilibration buffer (0.1 M Tris, 0.01 M citric acid, 0.075 M NaCl, pH 7.5) to remove the ethanol and a column was poured measuring 0.7 cm diameter x 2.5 cm in length (bed volume of 1 ml). The column was washed with 10 ml of equilibration buffer and when the ionicity of the column eluant was similar to that of the equilibration buffer the sample was applied to the column. The flow rate was adjusted to 0.75 ml/min and 0.5 ml fractions were collected. Following the application of the sample, the column was washed with equilibration buffer (approximately 10 ml) until the OD_{280nm} of fractions were the same as the equilibration

buffer. Proteins bound to the column were then eluted with a linear NaCl gradient using equal volumes (20 ml) of elution buffer (0.1 M Tris, 0.01 M citric acid, 2 M NaCl, pH 7.5) and equilibration buffer. After the gradient, an additional 15 ml of high salt buffer (0.1 M Tris, 0.01 M citric acid, 2 M NaCl, pH 7.5) was applied to the column to elute all tightly bound protein. Fractions were assayed for antibacterial activity by the standard microdilution growth assay. Those exhibiting antibacterial activity were pooled, dialyzed, concentrated by freeze-drying, and stored at -20°C until needed.

SDS-PAGE Analysis of Chromatography Fractions

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a rapid and reproducible way of analyzing proteins under reducing and non-reducing conditions, on the basis of their molecular size and mobility through gel matrix (Laemmli 1970). It is also a more accurate and dependable method than gel filtration for determining the molecular size of proteins/peptides of interest. Column fractions were analyzed by SDS-PAGE under reducing and non-reducing conditions to determine protein composition. Gels were either purchased commercially (gradient 10-20% Tris-Tricine Criterion gels) (*Bio-Rad, CA*), or poured in the laboratory (single percentage 7.5% and 10%). Samples were mixed in a 4:1 ratio with 5x sample buffer (with or without 5% mercaptoethanol), and 30 µl of each sample loaded into individual wells. Protein standards used as molecular weight markers consisted of a commercial mixture of proteins (*Bio-Rad, CA*) that included myosin (200 kDa), β-galactosidase

(116.3 kDa), phosphorylase B (97.4 kDa), BSA (66.3 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). Criterion gels (*Bio-Rad, CA*) were electrophoresed at room temperature at 200V (0.06 Amps) using chilled 1X Running buffer containing 0.1% SDS. Electrophoresis was stopped when the dye front migrated to the bottom of the gel. The gels were stained with Coomassie R-250 for 10 min with gentle agitation and destained for 24 hours in a solution containing 37.5% methanol, 10% acetic acid and 2.5% glycerol. Gels were then dried in a Model 450 gel dryer (*Bio-Rad, CA*), following manufacturer's instructions.

Western Blot

To identify whether proteins contained in active samples were histones/histone fragments, samples were analyzed in Western blots using heterologous antisera raised against a variety of human histone proteins. Western blots were set up as follows: following SDS-PAGE of samples, gels were equilibrated in transfer buffer for 15-20 min. Nitrocellulose membrane (*Bio-Rad, CA*) was first equilibrated with 100% methanol for 5 min, then added to the container with electrophoresed gel to equilibrate in transfer buffer for an additional 15 min. A "sandwich" of the gel and nitrocellulose membrane (in close contact and with no trapped air bubbles) between filter paper and sponge pads was carefully put together. Proteins from the gel were transferred to the membrane through electro-blotting by electrophoresing the sandwich for 1 hr at 100 volts.

Following transfer, the membrane was separated from the gel and immersed in blocking solution and incubated with gentle shaking for 90 min at room temperature followed by overnight incubation at 10°C. The blocking step was to ensure that all "sticky" sites on the membrane were blocked with milk protein contained in the blocking solution. After overnight incubation the blocking solution was decanted and the membrane washed with diluent buffer with gentle agitation, for 5 - 10 min at RT. The wash buffer was discarded and the membrane washed once more with wash buffer. Primary antibody reagent (10 ml) was added to the membrane and incubated at room temperature for 3 hr after which the primary antibody was removed and unbound antibody washed away from the membrane by adding more wash diluent buffer and incubating at RT for additional 20 min. This wash step was repeated once more. The wash buffer was discarded and the secondary antibody conjugate reagent (10 ml) was added and incubated at room temperature for 1 hr. After incubation, the secondary antibody reagent was decanted, and the membrane washed in buffer to wash away all unbound conjugate from the blot. The wash step was achieved by gently agitating the membrane for another 10 min at RT. The membrane was washed twice more. To develop the blot for visualization of reactive protein bands the membrane was immersed in 10 ml of HRP color development solution (*Bio-Rad, CA*) until color development was complete (approximately 2 - 5 min). Color development was stopped by immersing the membrane in distilled water for 5 min. Forceps were used to handle the membrane, placing it between two filter pads until dry and then wrapped in foil paper for storage at -20°C.

Correlation of Antibacterial Activity with Specific Protein Bands

To correlate antimicrobial activity detected in samples with a specific protein, active fractions were subjected to polyacrylamide gel electrophoresis under acid conditions, and the gel containing separated proteins/peptides was overlaid on an agarose seeded with *Planococcus citreus* (the underlay). The overlay permits protein bands to diffuse into the agarose and inhibit the growth of the bacterial lawn.

Acid-urea (AU) electrophoresis was performed using 12.5% acid-urea gels which were pre-run for 1 hr with reversed polarity at 30mA/120V using 5% acetic acid in both upper and lower buffer chambers. The running buffer was discarded and the gel assembly rinsed with ddH₂O and reassembled with fresh buffer. Test samples were diluted in 5% acetic acid, mixed with 3x sample buffer and immediately loaded into wells of two gels in identical order and run as before with fresh 5% acetic acid for 50 min at 80-100V at reverse polarity. After the gels were removed from the apparatus, one gel was stained with Laemmli coomassie stain while the other was rinsed three times with rinse buffer (changing rinse buffer every 5 min).

The washed gel was placed on an agarose underlay and the plates incubated at 30°C for 3 hr to permit diffusion of the protein/peptide bands into the underlay containing the target organism. The underlay was prepared by adding 100 µl of a standardized suspension of *Planococcus citreus* (approximately 10⁶ organisms/ml) to 10 ml of molten (44°C) 1% agarose, and allowing the mixture to solidify in a petri dish (100 x 15 mm square with 13 mm grid) at room temperature. Using the sterile tip of a

3mm diameter template cutter, two wells were punched into the agar along the periphery of the plate. 5 μ l each of positive control, HNP1 (25 μ g/ml) and negative control (3.2% saline solution) were then added to each respective well. Following incubation the gel was removed and stained (to determine efficiency of transfer) and the surface of each plate was overlaid with 10 ml of molten overlay nutrient medium necessary for bacterial growth (artificial sea water complete broth in 1% agar). Once the overlay had solidified, the plates were inverted and incubated at 30°C for 24 hr. The plates were examined for clear zones of inhibition of bacterial growth. The bacterial lawn appeared as a uniform turbid background. The plate was stained with the modified coomassie stain and photographed using a digital camera (*Sony*).

Amino Acid Sequence Analysis of Lysate Proteins/Peptides

Amino acid sequence data for specific proteins/peptides identified in lysate fractions was obtained either by Edman degradation (yielding N-terminal sequence) or by LC-MS/MS analysis of trypsin digests. Samples for analysis were lysate fractions in which antibacterial activity had been detected either by the agar diffusion assay or the standard microdilution growth assay with activity further correlated to specific protein band(s) by AU gel overlay assays.

Samples for N-terminal sequence analysis by Edman degradation were prepared as follows. Briefly, samples were subjected to SDS-PAGE, gels were equilibrated in transfer buffer containing 10% methanol for five minutes, and placed in a sandwich

containing the PVDF membrane in direct contact with the gel, and insulated with filter paper and fiber pads to ensure proper transfer. The sandwich was electrophoresed at 100V for 1hr (Criterion Gel System, *Bio-Rad*) and the membrane was removed from the sandwich and stained for 1 min in 0.02% Coomassie R-250 solution containing 40% methanol. It was then placed in destain solution of 40% methanol for up to 1 min and washed in distilled water for 3-5 min with at least three changes of water. Forceps were used to handle the membrane, placing it between two filter pads until dry and then wrapped in foil paper before mailing to the protein core facility (ICBR, University of Florida) for N-terminal sequence analysis. Protein bands that corresponded to those that (1) reacted in a Western blot with antisera to specific histones and/or (2) demonstrated antibacterial activity in acid urea overlay assays were chosen for sequencing.

Samples for trypsin digestion and LC-MS/MS analysis were prepared by subjecting them to SDS-PAGE analysis then gels were stained for 1 hour with Coomassie stain (*New England Biolabs, MA*), followed by destaining with water for 30 min. The bands selected for analysis were excised with a scalpel blade and placed in an eppendorf tube and stored at -20 °C until shipped in dry ice to the protein core facility (ICBR, University of Florida) for tryptic digestion and LC-MS/MS analysis.

The trypsin digested samples were injected onto a capillary trap (LC Packings, *PepMap*) and desalted for 5 min with a flow rate 3 µl/min of 0.1% v/v acetic acid. The samples were loaded onto an LC Packing® C18 Pep Map nanoflow HPLC column. The elution gradient of the HPLC column started at 3% solvent A, 97% solvent B and

finished at 60% solvent A, 40% solvent B for 30 min for protein identification. Solvent A consisted of 0.1% v/v acetic acid, 3% v/v ACN, and 96.9% v/v H₂O. Solvent B consisted of 0.1% v/v acetic acid, 96.9% v/v ACN, and 3% v/v H₂O. LC-MS/MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (*Fisher Scientific, GA*). The ion spray voltage was set to 2200 V. Full MS scans were acquired with a resolution of 60,000 in the orbitrap from m/z 300–2000. The five most intense ions were fragmented by collision induced dissociation (CID). Dynamic exclusion was set to 60 seconds.

Protein search algorithm

Data from all MS/MS samples were analyzed using Mascot (*Matrix Science, London, UK; version 2.2.2*). Mascot was set up to search both NCBI and NCBI_chondichthes databases assuming trypsin digestion. Mascot was searched with a fragment ion mass tolerance of 10 ppm and a parent ion tolerance of 10 ppm. Iodoacetamide derivative of Cys, deamidation of Asn and Gln, oxidation of Met, are specified in Mascot as variable modifications. Scaffold (*version Scaffold-03-00-04, Proteome Software Inc., Portland, OR*) was used to validate MS/MS based peptide and protein identifications. Peptide identifications are accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications are accepted if they can be established at greater than 99.0% probability and contain at least two identified unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm.

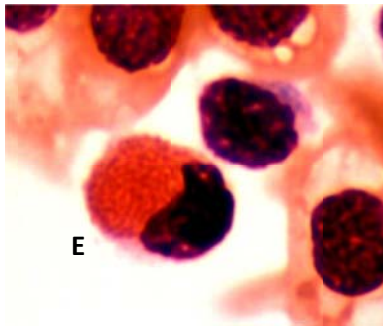
IV. RESULTS

Composition of Leukocyte Fraction of Shark Peripheral Blood

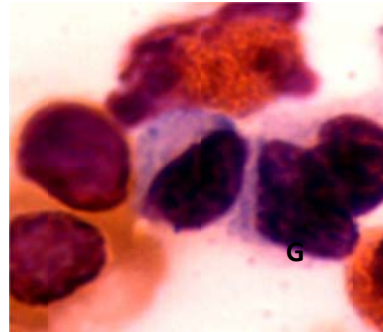
Structural morphology of cell types

Since the aim of the study is to identify proteins or peptides associated with antibacterial activity of leukocytes, it was essential to confirm that lysates made from leukocytes were made up of all leukocyte types. Figure 1a through 1e show different cell types found in nurse shark peripheral blood leukocytes. Two major granulocytic cells are present. One is eosinophil-like, a granulocyte with deeply staining eosinophilic intracytoplasmic granules with a lobed nucleus (Figure 1a). These cells are similar in staining properties to mammalian eosinophils. The second type of granulocytic cell is the neutrophil-like granulocyte, characterized by an eccentric, usually bi-lobed, nucleus surrounded by cytoplasm containing granules (Figure 1b), distinctly different from those of the eosinophilic granulocyte. Four types of mononuclear cells are present. The monocyte-macrophage cells are mononuclear and lack distinct granules. The nucleus is large and usually no more than half of the cell volume (Figure 1c). The second mononuclear cell is the lymphocyte-like cell (Figure 1d), which is similar to a mammalian lymphocyte with a spheroidal nucleus occupying much of the cell volume, and surrounded by a narrow rim of cytoplasm. The third cell type is the thrombocyte, which usually appears discoidal with a single, large, centrally placed, oval nucleus (Figure 1e). However, depending on how the blood is processed, thrombocytes may assume several distinct shapes (e.g., spiked, spindle or fragmented). Another cell type occasionally

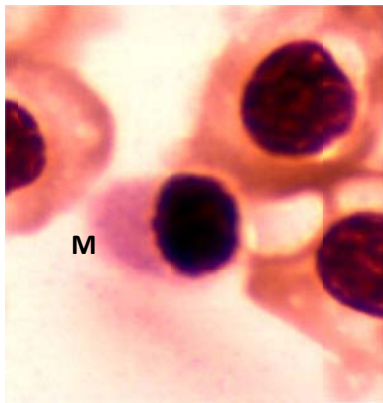
found in peripheral blood is the undifferentiated blast cell (not shown). This cell type exhibits an undistinguished cytoplasm with a central nucleus.



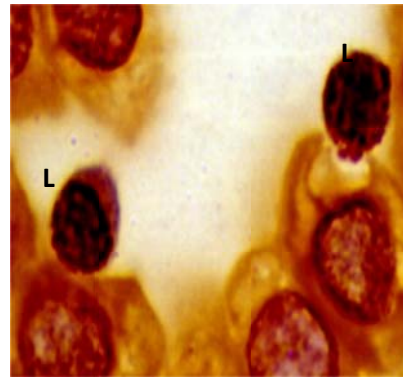
(a)



(b)



(c)



(d)



(e)

Figure 1 Cytospins of nurse shark peripheral blood cells. Cytospins made using heparinized nurse shark peripheral blood on poly-L-Lysine coated slides and stained with leukostat. (a) eosinophil-like, **E**. (b) neutrophilic granulocyte, **G**. (c) monocyte-macrophage, **M**. (d) lymphocyte-like, **L**. (e) thrombocytes, **T**.

Differential count of cell types

Differential leukocyte counts (carried out in duplicate) of peripheral whole blood, from one individual, yielded relative numbers of the cell types. These are shown in Table 1, expressed as percentage, with 200 leukocytes counted per slide (average of three slides). A normal differential leukocyte count of human peripheral blood, expressed as percentage, is provided for comparison (Altman et al. 1961).

Table 1 Relative Numbers of Different Leukocytes in Nurse Shark (present study) and Human Peripheral Blood

Nurse Shark Peripheral Blood		Human Peripheral Blood	
Cell Type	%	Cell Type	%
Neutrophilic granulocyte	32.5	Neutrophilic granulocyte	59
Eosinophil-like	11.5	Eosinophil	2.7
Monocyte-macrophage	2.5	Monocyte-macrophage	4
Lymphocyte-like	40	Lymphocyte	34
Basophil	0	Basophil	0.5
Thrombocyte	13.5	Thrombocyte	n/a

Detection and Quantitation of Antibacterial Activity of Leukocyte Lysates

Lysates were prepared as stated in the materials and methods. Protein concentration of lysate preparations ranged from 2-8 mg/ml, depending on the number of leukocytes harvested and the procedure used to disrupt cells. Initial screening of crude lysates for lysozyme and antibacterial activity was performed using the lysoplate

and agar diffusion methods, respectively (Osserman et al. 1966; Lehrer et al. 1991; Manchenko 1994). Antibacterial activity against *Planococcus citreus* was detected in all lysates tested, except for lysate sample #3 at 25°C (Table 2).

Table 2 Quantitation of Antibacterial Activity of Different Preparations of Leukocyte Lysates

Lysate sample	Antibacterial Activity in AU	
	At 30°C	At 25°C
Leukocyte lysate sample #1	35	25
Leukocyte lysate sample #2	50	40
Leukocyte lysate sample #3	5	0
Leukocyte lysate sample #4	40	15
Leukocyte lysate sample #5	45	30
Leukocyte lysate sample #6	80	60

NOTE: Activity Units (AU) represents the average of duplicate readings at each temperature

The modified radial diffusion assay supplemented with sea water complete medium (SCM) was semi-quantitative and antibacterial activity was estimated by measurement of the diameter of the zones of inhibition as seen in Figure 2. The level of antibacterial activity varied from preparation to preparation as expected, because lysates were prepared from different blood samples, collected at different times. Antibacterial assays carried out at 30°C showed larger and more pronounced zones of inhibition than

assay plates incubated at 25°C (Figure 2a and b), using similar samples. Antibacterial activity was expressed in arbitrary activity units (AU) calculated from the diameter of the zone of inhibition (for details see Methods) (Table 2) according to the formula:

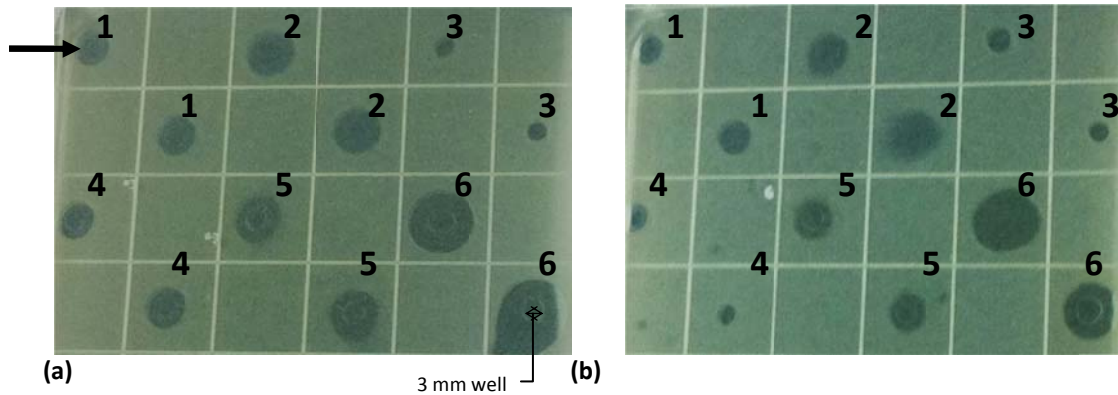
$$\text{Arbitrary activity units} = (\text{Diameter of zone of inhibition (mm)} - \text{Diameter of well (mm)}) \times 10$$


Figure 2 Representative radial diffusion assay plates. Antibacterial activity is seen as a clear area (arrow) around wells (wells are 3mm in diameter). Duplicate samples (10 μ l) of six different lysate preparations (1-6 in figure) were individually added to wells. Duplicate plates were incubated (a) at 30°C and (b) at 25°C. Diameter of the zones was measured after 24 h incubation and antibacterial activity expressed in activity units calculated according to the formula in materials and methods (Table 2).

When lysates were prepared from different bleeds, and antibacterial activity was measured at 25°C and 30°C, it was seen that activity, as shown by zones of inhibition measured (Figure 2a, arrow), was consistently higher when plates were incubated at 30°C as opposed to 25°C.

When assaying for lysozyme-like activity using the lysoplate assay, retention of activity was inconsistent and was affected by the method used to prepare the extracts, i.e., mechanical shearing, homogenization versus sonication. When the method of preparation was mechanical shearing or glass homogenization, the lysozyme-like activity

was detected. However, when the method of preparation was sonication, the lysozyme-like activity could not be detected by the lysoplate assay.

Development of a microdilution growth assay

The radial diffusion assay was effective as an initial screening method, however, a more sensitive and accurate assay, such as a microdilution growth assay (not currently available for sharks) was necessary.

In order to develop this assay, optimal conditions (pH, salinity, incubation temperature, etc.) for growth of the target organism, *P. citreus*, and for antibacterial activity (growth inhibition) was determined.

(1) Growth curve of *P. citreus* in SCM

Growth of *Planococcus citreus* in SCM was monitored by measuring OD at 570 and 630 nm in micro-well plates over a period of 24h at 30°C (Figure 3). The culture reached mid-log phase in 10 hours. No significant difference was noted between OD readings taken at 570 or 630 nm, and subsequent experiments measuring bacterial growth were read at 570 nm. Ten h cultures (growing exponentially) were used as target cell inoculum for all future microdilution growth assays.

(2) Effect of pH and salinity on *P. citreus* growth

Microdilution growth assays were set up in SCM medium adjusted to varying pH (4.0, 5.5, 6.5, 7.5 and 8.5) and salinity (0.3M, 0.4M, 0.5M, 0.6M NaCl). Since previous radial diffusion assays had shown enhanced antibacterial activity at 30°C, microdilution growth assays were incubated at 30°C. The data shown in Table 3 are represented by OD_{570nm}. Growth at pH 4.0, 5.5 and 6.5 was minimal. Growth did

occur at pH 7.5 and 8.5. For the standard assay, a pH of 7.5 was chosen as it is closer to physiological pH for shark leukocytes. *Planococcus citreus* grew at identical rates in SCM adjusted to a final NaCl concentration of 0.3M, 0.4M, 0.5 and 0.6M NaCl. Since the concentration of NaCl in SCM is normally 0.5M, all assays were performed using SCM without adjusting salt concentration.

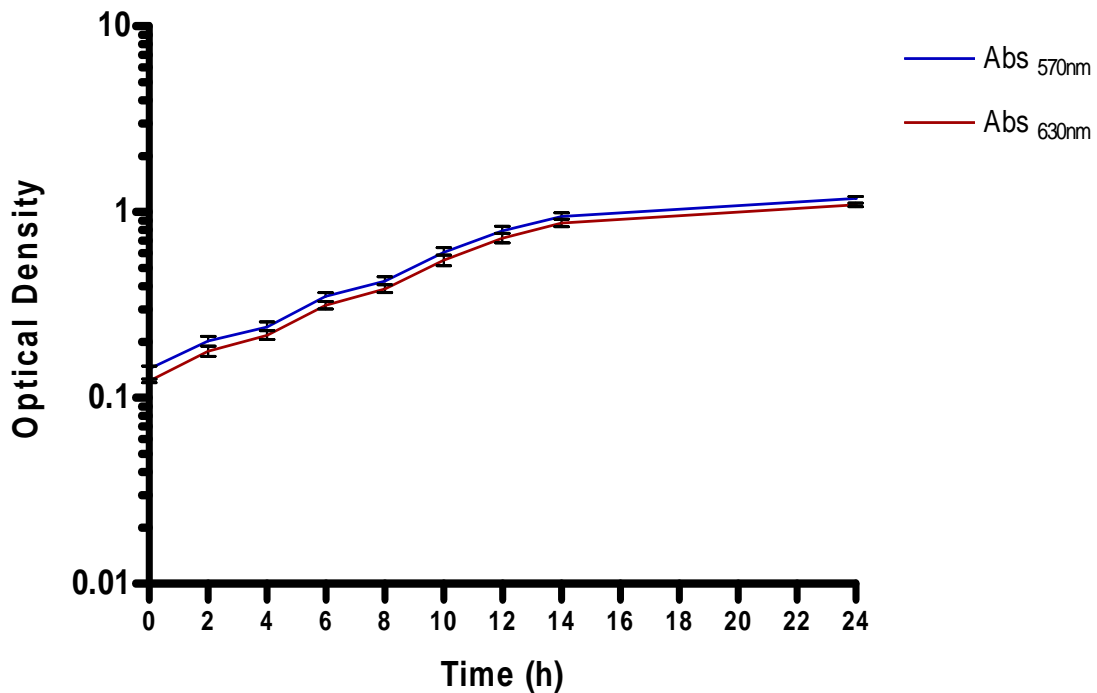


Figure 3 Growth of *Planococcus citreus* in Sea water Complete Medium. A 10 h culture (50 μ l) of *P. citreus* in sea water complete medium (SCM) was inoculated into 50 μ l fresh 2x SCM (salt concentration was unchanged (1x) in double strength media). The culture was incubated at 30°C and OD read at 2h intervals in a Dynex ELISA reader at wavelength 570 and 630 nm. Each point represents an average OD reading of samples from triplicate cultures. Error bars fall within plot points and are not visible.

Table 3 Effect of pH and Salinity on Growth of *Planococcus citreus*

pH of media	OD	
	0 h	12 h
4.0	0.004	0.013
5.5	0.004	0.021
6.5	0.003	0.055
7.5	0.007	0.326
8.5	0.004	0.280

(a)

NaCl concentration of media	OD	
	0 h	12 h
0.3M	0.003	0.307
0.4M	0.004	0.285
0.5M	0.009	0.321
0.6M	0.010	0.312

(b)

NOTE: A 10 h culture (100µl) of *P. citreus* in SCM was inoculated into 75 µl fresh 2x SCM, adjusted to (a) different pH and (b) different salinity. The culture was incubated at 30°C and OD_{570nm} read at 2h intervals in a Dynex ELISA reader. Each point represents an average OD reading of samples from duplicate cultures.

(3) Determination of optimal temperature for *P. citreus* growth and antibacterial activity of lysate for microdilution growth assay

Optimal incubation temperature was determined by monitoring growth of *P. citreus* with and without the lysate at 30°C and 25°C (Table 4). Thirty degree celsius was optimal for growth of the target organism and maximal for antibacterial activity of the lysate.

Table 4 Effect of Temperature on *P. citreus* Growth and Antibacterial Activity of Leukocyte Lysate

Incubation temperature	OD	
	0 h	12 h
25°C	0.013	0.036
30°C	0.011	0.119
25°C + lysate	0.032	0.052
30°C + lysate	0.032	0.048

NOTE: *Planococcus citreus* cultures were incubated with and without lysate and incubated at 25°C and 30°C. Bacterial growth was measured by taking OD_{570nm} readings at 2h intervals in a Dynex ELISA. Each reading represents an average OD reading from triplicate cultures.

(4) Determination of optimal target cell density for microdilution growth assay

The optimal concentration of *Planococcus citreus* for the assay was determined by performing the assay using two different concentrations of bacteria; 1×10^5 CFU/ml and 2.5×10^4 CFU/ml. The effect of crude lysate, concentrated four-fold, on *Planococcus citreus* growth was followed over a 24 hr period at 30°C. At 10 hours no significant effect (antibacterial) on growth of *P. citreus* was noted at a cell density of 1×10^5 CFU/ml (Figure 4). Lack of activity may be caused by excessive cell density, masking any inhibitory effect. Antibacterial activity was measurable as inhibition of growth after 4 hours, at a cell density of 2.5×10^4 CFU/ml. This was the density of target cells used for all future assays.

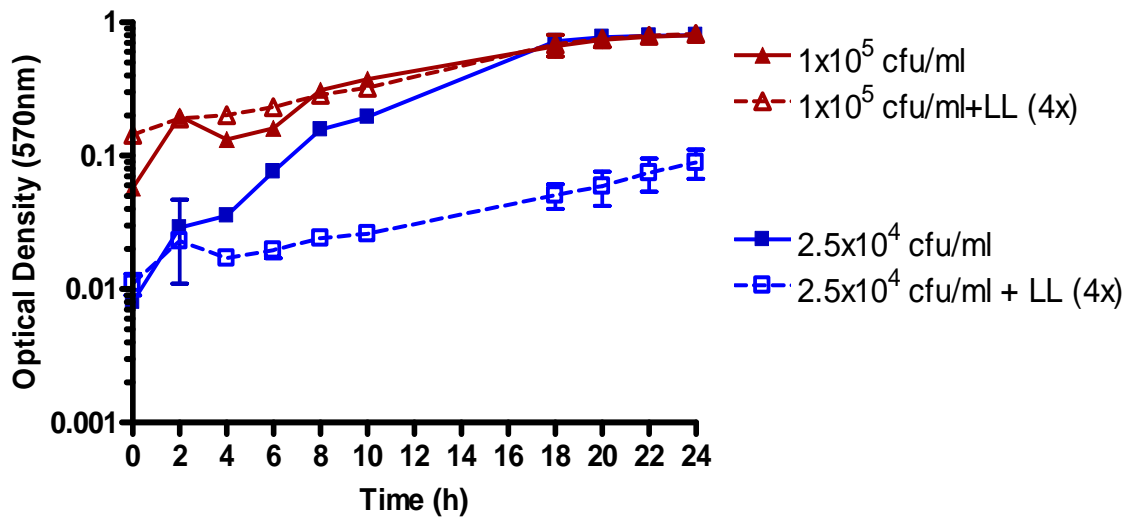


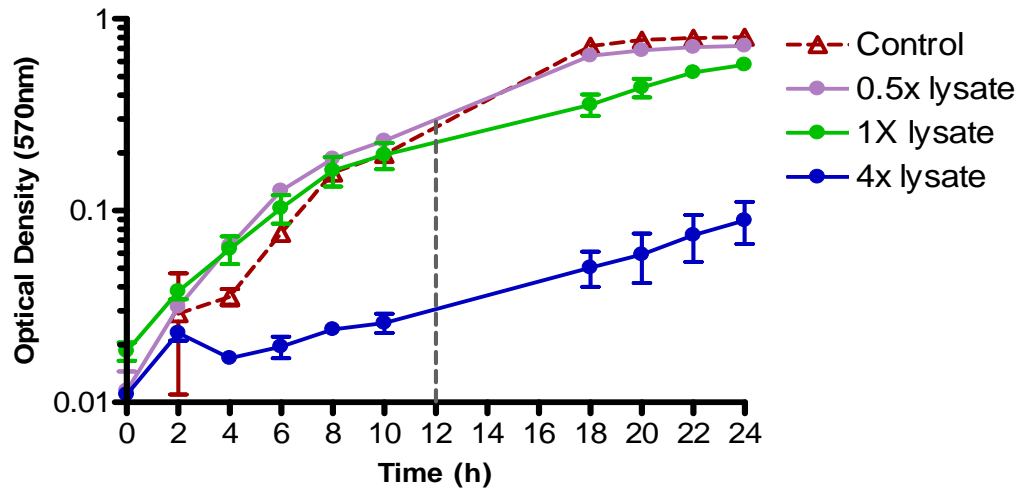
Figure 4 Antibacterial effect of leukocyte lysates on growth of *Planococcus citreus* at different target cell densities. A four-fold concentration of the crude extract was added to different densities of *P. citreus* (1×10^5 CFU/ml and 2.5×10^4 CFU/ml) and bacterial growth at 30°C was compared to corresponding control culture consisting of *P. citreus* in the absence of lysate. Each reading represents an average OD_{570nm} of samples from triplicate cultures (measured at 2 h intervals for 24 h).

(5) Sensitivity of microdilution growth assay

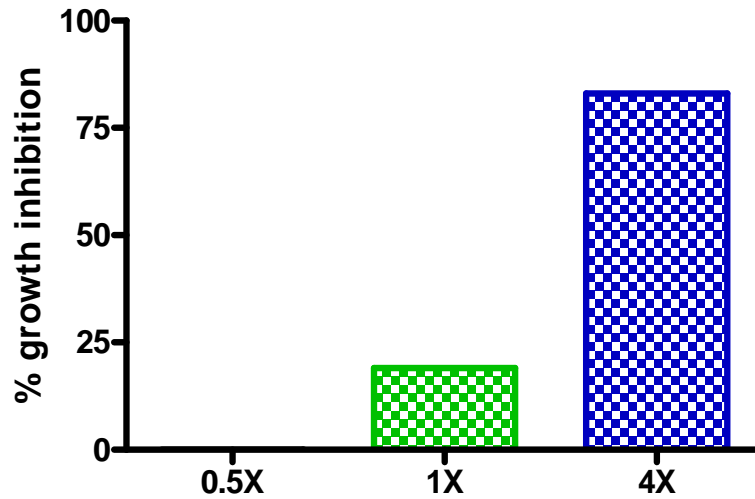
The minimum threshold level of antibacterial activity detectable (in crude lysate) by micro-titration was determined by measuring the antibacterial effect of different concentrations of lysate on growth of *P. citreus*. A crude lysate (1x), a two-fold diluted (0.5x) sample and a four-fold concentrated sample of the same lysate (4x) were compared for growth inhibition (Figure 5). There was no appreciable difference in bacterial growth when the culture with lysate, diluted two-fold (0.5x), was compared to the control culture. However, inhibition of bacterial growth was shown by the crude lysate (1x) after 10 hours, and by the 4x concentrated lysate by 4 hours. Results indicate that the antibacterial activity of crude lysates can be measured by the assay after 10 hours of incubation. The end point for the standard assay was set for 12 hours (Figure 5b).

(6) Establishment of standardized microdilution growth assay

On the basis of the results above, the microdilution growth assay was standardized as follows. Each assay consisted of 50 µl of test sample, 75 µl of 2x SCM and 100 µl standardized *P. citreus* inoculum (2.5×10^4 CFU/ml, final concentration). The SCM contained 0.5M NaCl and pH was adjusted to 7.5. Assays were set up in triplicate and incubated at 30°C for up to 12 hours. Wells were read at OD_{570nm}.



(a)



(b)

Figure 5 Antibacterial activity of diluted and concentrated samples of crude leukocyte lysate. (a) A two-fold diluted sample, a crude lysate, and a four-fold concentrated sample of the same LL was added to *P. citreus* at a cell density of 2.5×10^4 CFU/ml and bacterial growth at 30°C was compared to corresponding control cultures consisting of *P. citreus* in the absence of lysate. Each point represents an average OD of samples from triplicate cultures. (b) Percent growth inhibition of a two-fold diluted sample, a crude lysate, and a four-fold concentrated sample of the same LL calculated at 12 h.

Application of standard assay to titrate antibacterial activity in lysates

To quantify the level of antibacterial activity present in samples, microdilution growth assays were set up using three different lysate preparations (lysate preps were from different blood samples, drawn at different times from different animals). Two-fold serial dilutions were made of each lysate in SCM and assayed for antibacterial activity, which was measured and expressed as percent growth inhibition calculated according to the formula:

$$\% \text{ Inhibition} = \frac{\text{Final OD}_{570\text{nm}} \text{ } P. \text{ citreus} \text{ control} - \text{Final OD}_{570\text{nm}} \text{ of test sample}}{\text{Final OD}_{570\text{nm}} \text{ } P. \text{ citreus} \text{ control}} \times 100$$

where 'test sample' is *P. citreus* with test leukocyte lysate (LL).

Figure 6 shows that with increasing dilution of test lysate a corresponding decrease in antibacterial activity (measured as percent inhibition of *P. citreus* growth) is noted. The assay has sufficient sensitivity to detect antibacterial activity in crude lysate samples containing varying levels of antibacterial activity. Lysate sample, LL-1S was the least active lysate, while LL-3S showed the most antibacterial activity. The antibacterial activity titer of a lysate was taken as the inverse of the highest dilution that inhibited growth of *P. citreus*. Thus the antibacterial activity titer of LL-1S, 2S and 3S is 4, 8 and greater than 16, respectively. However, the growth inhibition detected below 20% may not be meaningful after an eight-fold dilution.

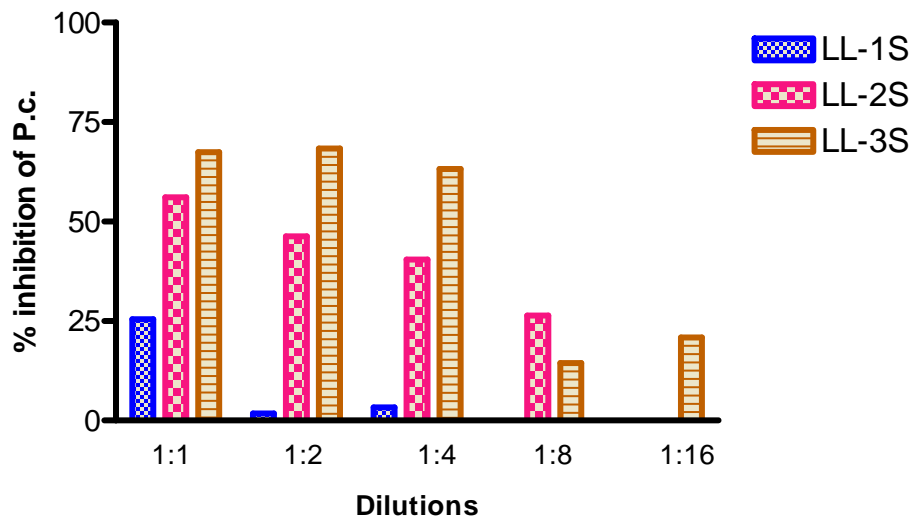


Figure 6 Variation in the antibacterial activity of different lysate preparations from different bleeds. Serial two-fold dilutions of lysates, LL-1S, LL-2S and LL-3S were made in 2x SCM. The assay was incubated at 30°C and assay end point OD_{570nm} readings taken at 12 h in a Dynex ELISA reader. Each bar represents percent inhibition calculated from mean OD of duplicate cultures and compared to the control (*P. citreus* in the absence of lysate).

Fractionation of Cell Lysates and Protein Analysis of Active Fractions

The initial screening of lysates for antibacterial activity was by the agar diffusion assay method. Lysates prepared by mechanical shearing, homogenization or sonication were fractionated by ion-exchange chromatography and several different permutations of chromatographic separation were employed. Column fractions were assayed for antibacterial and lysozyme activity. Fractions exhibiting antibacterial activity were further analyzed by SDS-PAGE analysis to determine protein composition and select protein bands were selected for amino acid sequence analysis.

Protocol I: DE Cellulose-Heparin

Protocol I consisted of a two step fractionation on DE cellulose, followed by affinity chromatography (Figure 7-9). Profiles of ion-exchange chromatography are illustrated in

Figures 7 and 8. Briefly, a lysate prepared by mechanical shearing using a glass homogenizer was concentrated four-fold by ultra-filtration and applied to the first DE-52 cellulose column. Fractions were assayed for lysozyme and antibacterial activity. Most of the lysozyme activity was recovered in the pre-gradient fractions along with some antibacterial activity (Figure 7). Some lysozyme activity was also recovered in early post gradient fractions. Most of the antibacterial activity, however, was present in the post-gradient fractions (Figure 7). The active fractions were pooled into 4 distinct pools consisting of the following fractions: Pool I (6-12), Pool II (70-77), Pool III (78-87), Pool IV (103-115) and Pool V (116-125). Pool I was concentrated by ultra-filtration and applied to a second DE cellulose column run under similar conditions to separate lysozyme from antibacterial activity. A similar elution profile to that of the first column was obtained (Figure 8) with lysozyme present in pre- and early post-gradient fractions, and with antibacterial activity separated and found in the later post-gradient fractions.

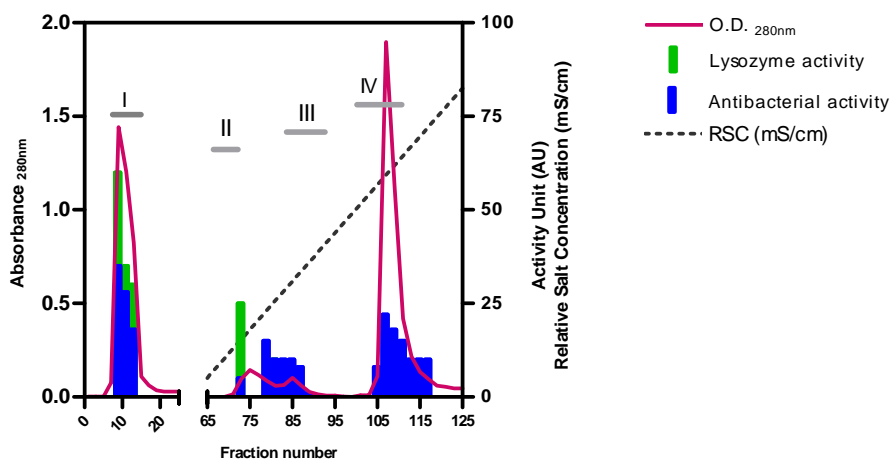


Figure 7 Protocol I: Fractionation of leukocyte lysate on DE Cellulose. Crude lysate (2.5 ml) mixed with an equal volume of equilibration buffer was applied to DE-52 column (1.0 x 4.5 cm) equilibrated with 0.02M Tris-HCl, pH 7.5. Fractions (0.8 ml) were collected at a flow rate of 0.7 ml/min at 25°C. Bound proteins were eluted from the column with a linear NaCl gradient, up to 0.9M NaCl. Antibacterial (blue bars) and lysozyme activity (green bars) were measured by agar diffusion for every other fraction and recorded as activity units (for details see Methods).

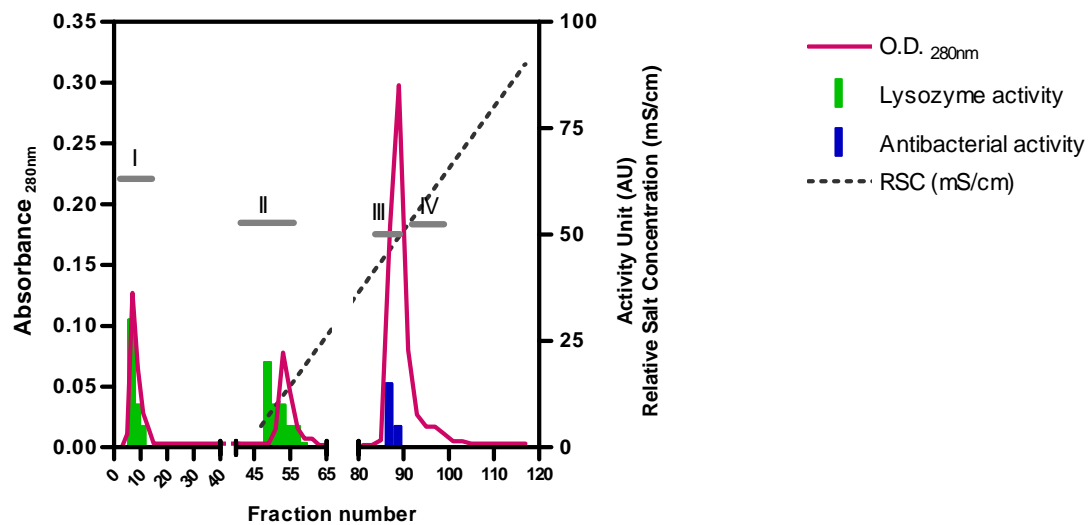


Figure 8 Protocol I: Fractionation of Pool I from the first DE Cellulose column. Crude lysate (2 ml) mixed with an equal volume of equilibration buffer was applied to DE-52 column (1.0 x 4.5 cm) equilibrated with 0.02M Tris-HCl, pH 7.5. Fractions (0.8 ml) were collected at a flow rate of 0.7 ml/min at 25°C. Bound proteins were eluted from the column with a linear NaCl gradient, up to 0.9M NaCl. Antibacterial (blue bars) and lysozyme activity (green bars) was measured by agar diffusion for every other fraction and recorded as activity units (for details see Methods).

Active fractions of the second column were pooled into four pools: Pool I (6-12), Pool II (50-55), Pool III (84-89) and Pool IV (90-93). Pool IV and Pool III, containing most of the antibacterial activity from the first and second chromatographic separations, respectively, were combined and dialyzed against the equilibration buffer (100mM Tris-HCl, 10mM citric acid, 225mM NaCl, pH 7.4) used for heparin affinity chromatography. Ten ml of the combined pool was applied to a heparin column (Figure 9a). Bound proteins were eluted by batch elution using buffers varying in salt concentration from 0.225M to 1.5M NaCl. Fractions were screened for antibacterial activity and active fractions were pooled (Fractions 79-89), concentrated five-fold and further analyzed by SDS-PAGE analysis (Figure 9b). Proteins were subsequently transferred to a PVDF membrane for sequence analysis. An SDS-PAGE gel stained with coomassie blue

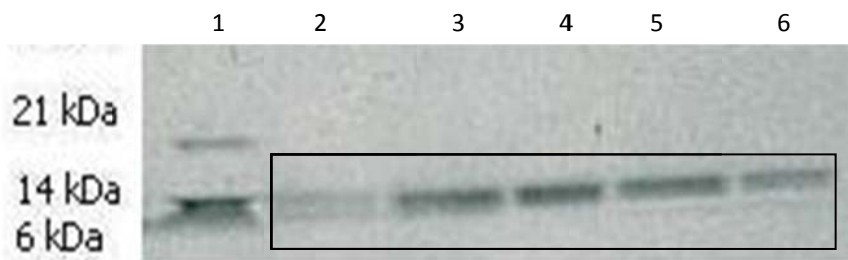
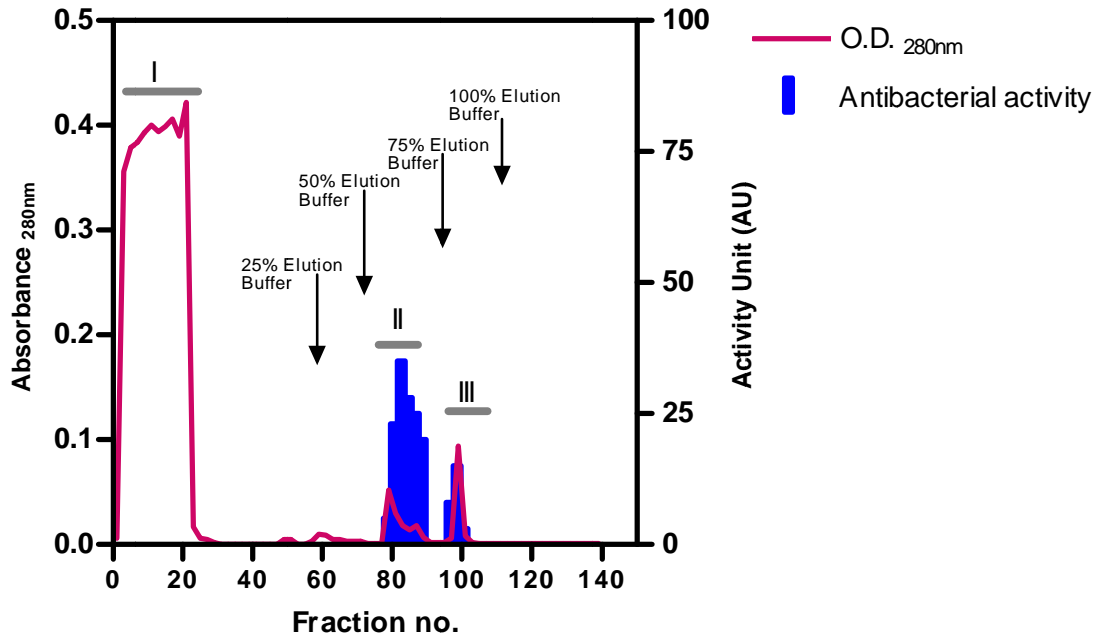
revealed two distinct protein bands between 6 and 14 kDa (Figure 9b). Protein bands were subjected to Edman Degradation for N-terminal protein sequencing (performed by University of Florida Protein Core). The upper band (~14 kDa) generated an N-terminal sequence of 14 residues with homology to teleost histone, H2B (see Table 5 below). The lower band (~6 kDa) generated an N-terminal sequence of 14 residues with homology to teleost histone, H2A (see Table 5 below). Pool I, which contained only lysozyme activity, from the second chromatographic separation was similarly loaded onto a heparin affinity column, but no bound protein was eluted (profile not shown).

Protocol IIA: DE Sepharose-glass homogenized lysate

An alternative fractionation protocol consisted of a single fractionation step on DE – Sepharose. A leukocyte lysate was prepared by mechanical shearing and concentrated ten-fold by using a combination of vacuum centrifugation and ultra-filtration (membrane cut-off MWCO, 3.5kDa). The sample (800 µl) was dialyzed against the column equilibration buffer (0.02M Tris-HCl, pH 7.5) and applied to a DE Sepharose column. Bound proteins were eluted with a linear gradient, up to 0.9M NaCl (Figure 10a). Pre- and post-gradient fractions showing peak antibacterial activity were pooled. Pre-gradient and early post-gradient fractions, Pool I (9-19) and Pool III (61-75) contained antibacterial, along with lysozyme activity which was absent from the later post-gradient fractions, Pool IV (95-107) and Pool V (108-121) that contained only antibacterial activity. Control assays testing elution buffers corresponding to the salt concentration at the end of the gradient showed inhibition of growth. Therefore, Pool V

was not processed further since growth inhibition was attributed to the presence of high salt and not protein.

(a)



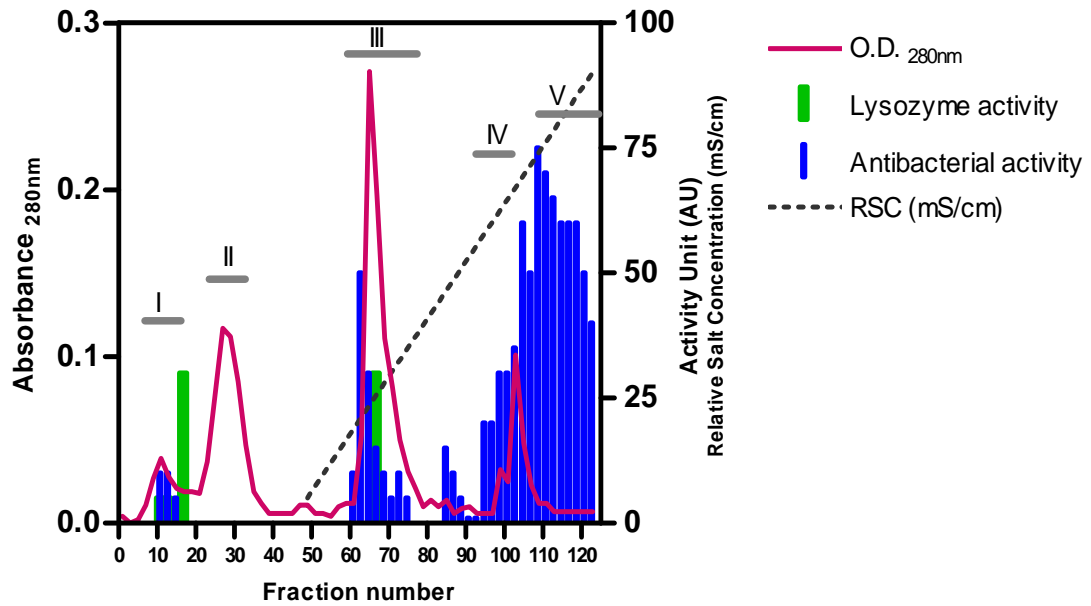
(b)

Figure 9 Protocol I: Fractionation of the combined pools from two DE Cellulose columns (see text) on Heparin Affinity column and SDS-PAGE analysis. (a) Combined pooled sample (10 ml) was applied to Heparin Affinity column (0.7 x 2.5 cm) equilibrated with 100mM Tris-HCl, 10mM citric acid, 225mM NaCl, pH 7.4. Fractions (0.5 ml) were collected at a flow rate of 0.75 ml/min at 25°C. Bound proteins were eluted step-wise using equilibration buffer diluted 25%, 50%, 75% and 100% with elution buffer (100mM Tris-HCl, 10mM citric acid, 1.5M NaCl, pH 7.4). Antibacterial activity (blue bars) was measured by agar diffusion for every other fraction and recorded as activity units (for details see Methods). Post-gradient fractions (fr 79-89) were pooled (Pool II) and analyzed by SDS-PAGE (b) Lanes 2 through 6 contained β mercapto-ethanol reduced samples of Pool II. Molecular weight standard (MWS) (Bio-Rad Broad Range) was run in lane # 1.

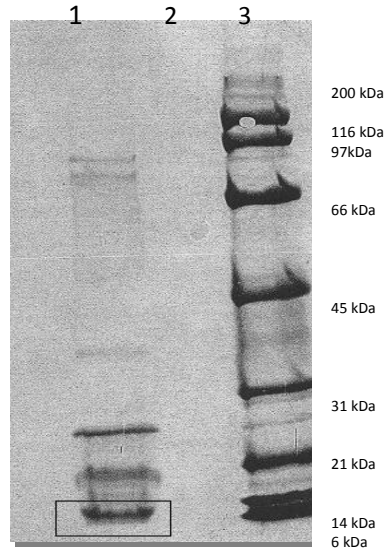
Pool IV (fractions 95-107) was concentrated ten-fold by ultra-filtration (MWCO 3.5kDa). Pool IV was further analyzed for protein composition using SDS-PAGE analysis (Figure 10b). Proteins from the gel were subsequently transferred to a PVDF membrane for sequence analysis. The lowest band (~6 kDa) was subjected to Edman Degradation for protein sequencing (University of Florida Protein Core). The protein band generated an N-terminal amino acid sequence of 12 residues with homology to teleost and mammalian histone, H2A (see Table 5 below). The upper bands were also sequenced but yielded amino acid sequences with no significant homology.

Table 5 N-terminal Amino Acid Sequence of Proteins Isolated from Shark Leukocyte Lysate Analyzed by Edman Degradation

Sample	Band	Sequence	ID
Pool II- Heparin Affinity (Protocol I)	Upper	KGAKKRSRSRKEXY	Histone H2B
Pool II -Heparin Affinity (Protocol I)	Lower	SIAISRSYRAGLQF	Histone H2A
Pool IV-DE Sepharose (Protocol IIA)	Lower	SRSERAGLQFQK	Histone H2A



(a)



(b)

Figure 10 Protocol IIA: Fractionation of leukocyte lysate on DE Sepharose and SDS-PAGE analysis. (a) Crude lysate (0.8 ml) mixed with an equal volume of equilibration buffer was applied to DE Sepharose column (1.0 x 4.5 cm) equilibrated with 0.02M Tris-HCl, pH 7.5. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min at 25°C. Bound proteins were eluted with a linear NaCl gradient, up to 0.9M NaCl. Antibacterial (blue bars) and lysozyme activity (green bars) was measured by agar diffusion for every other fraction and recorded as activity units (for details see Methods). Post-gradient fractions (95-107, pool IV) were pooled, concentrated ten-fold and analyzed by SDS-PAGE. (b) Lane 1 contained Pool IV. Lane 2 contained sample buffer only. Molecular weight standard (MWS) (Bio-Rad Broad Range) was run in lane # 3.

Fractionation of Cell Lysates and Correlation of Antibacterial Activity with Specific Protein Bands

Since histone fragments and other types of protein fragments and peptides have been shown in other species to have antibacterial activity, experiments were performed to determine whether antibacterial activity could be correlated to specific protein band(s) and to show whether the shark histone fragments identified were antibacterial. Thus, subsequent fractionation was carried out employing similar chromatographic conditions (see above) with minor modifications to buffers. Also fractions were screened for antibacterial activity using the standard microdilution growth assays in place of the agar diffusion assay. In addition, lysates were prepared by sonication rather than mechanical shearing and/or homogenization, since a higher level of activity was recovered in the sonicated lysate. Several DE Sepharose columns were run either singly or in series to fractionate the leukocyte lysates. To correlate antimicrobial activity with a specific protein/peptide, active fractions and/or pools were subjected to the acid-urea gel overlay protocol.

Protocol IIB: DE Sepharose-sonicate lysate I

A leukocyte lysate prepared by sonication of leukocytes and concentrated five-fold by lyophilization, was reconstituted in 0.5 ml ddH₂O and applied to a DE Sepharose column. Figure 11 shows the chromatographic profile yielding two major protein peaks, a pre-gradient and post-gradient peak. Every other fraction was assayed for antibacterial activity using the microdilution growth assay and antibacterial activity was

present in both pre-gradient and post-gradient fractions. The peak activity fractions were further analyzed for protein composition using SDS-PAGE.

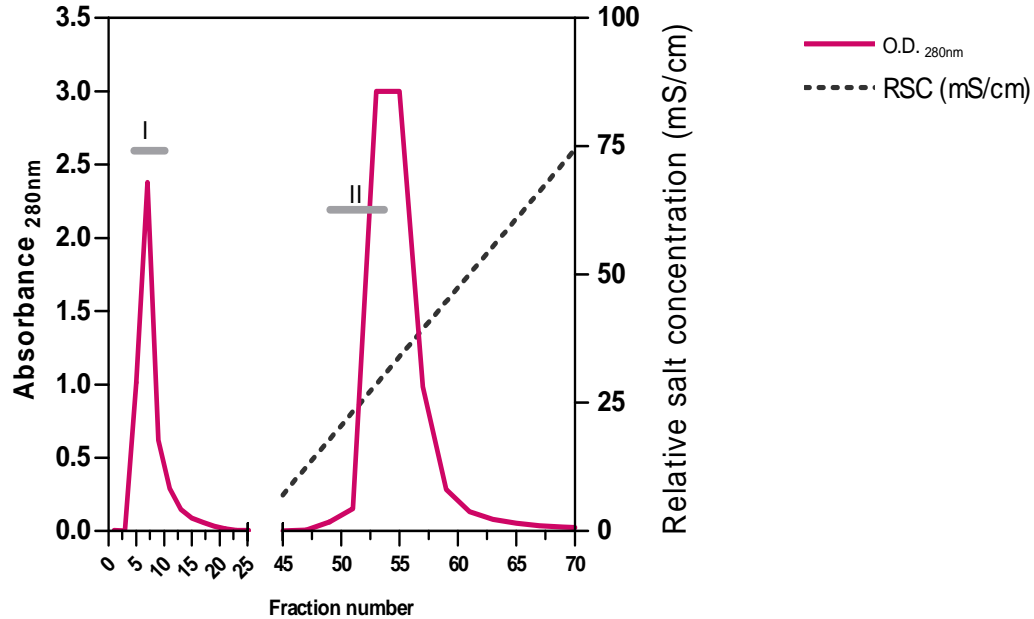


Figure 11 Protocol IIB: Fractionation of lysate on DE Sepharose column. A five-fold concentrated crude lysate (0.5 ml) mixed with equilibration buffer (0.3 ml) was applied to DE Sepharose column (1.0 x 4.5 cm) equilibrated with 0.02M Tris-HCl, 0.05M NaCl, pH 7.5. Fractions (1 ml) were collected at a flow rate of 1 ml/min at 25°C. Bound proteins were eluted from the column with a linear NaCl gradient, up to 0.9M NaCl.

Fractions 4-9 were pooled (Pool I), concentrated ten-fold by lyophilization, and subjected to acid-urea gel electrophoresis and analyzed for antibacterial activity by applying to a lawn of target organisms (acid-urea overlay). Distinct zones of inhibition were seen in the seeded lawn (Figure 12a) corresponding to the position of protein bands in the AU transfer gel. The acid-urea transfer gel stained with coomassie blue (Figure 12b), shows residual protein remaining after transfer of the 'active' protein to the agar overlay plate seeded with *P. citreus*. The position of residual protein bands correlates with the zone of inhibition noted on acid-urea overlay agar plate.



Figure 12 Acid-Urea PAGE analysis of concentrated Pool I (Protocol IIB). (a) Concentrated Pool I, from Protocol IIB in lane 1, was loaded onto a pre-run gel and electrophoresed. Gel was washed in 10mM phosphate buffer pH 7.5 and overlaid on agar seeded with *Planococcus citreus* and incubated 3 h at 30°C. Gel was removed and SCM poured on top and allowed to solidify. Plates were incubated overnight at 30°C. (b) Acid-Urea transfer gel. Transfer gel was removed from seeded agar and stained with coomassie brilliant blue R-250 for 1h and destained overnight to confirm that protein had transferred to the agar. Residual protein remained in the gel, however most protein was transferred. The position of the partial protein bands correlated to the position where inhibition zones were noted in the agar.

Pool I was further analyzed for protein composition using SDS-PAGE analysis. Protein bands were subsequently excised from the gel for sequence analysis (Figure 13a). The intense band (~20 kDa) was subjected to trypsin digest, followed by LC-MS/MS analysis (performed by University of Florida Protein Core). The protein band generated an internal amino acid sequence of three unique fragments, 9, 13 and 16 residues long with homology to human polyubiquitin (Table 6). An attempt was made to further separate the intense band by using a gel with a smaller pore size, 12% (Figure 13b) vs. 8% (Figure 12a). The resulting gel showed two distinct lower bands, similar to the bands seen in the acid urea gel, Figure 13b, these lower bands were excised (Figure 13, arrows), and sent for trypsin digest and LC-MS/MS analysis. Band 1 yielded a unique 6 residue fragment with sequence homology to ubiquitin from the catshark (see Table 6). Band 2 yielded a 5 residue fragment with sequence similarity to nurse shark nucleoside diphosphokinase, but had less than 80% probability and only one unique peptide.

Protocol IIC-1: DE Sepharose-sonicate lysate II

Another leukocyte lysate prepared by sonication and concentrated three-fold by lyophilization and reconstituted in 0.5 ml ddH₂O was applied to a DE Sepharose column. The chromatographic profile (Figure 14) shows three protein peaks, a pre-gradient and two post-gradient peaks.

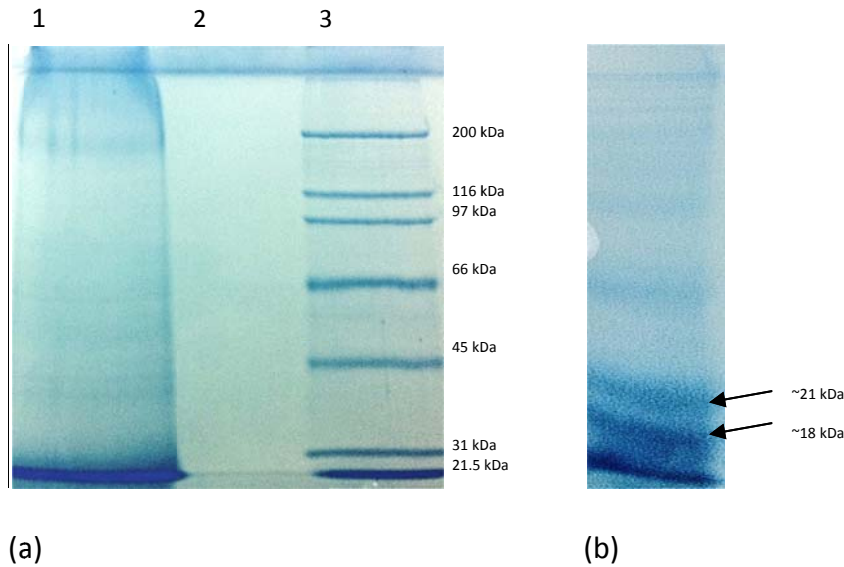


Figure 13 SDS-PAGE Analysis of Pool I from DE Sepharose column (Protocol IIB). Fractions were analyzed using SDS-PAGE. (a) Pool I (DE Sepharose-Protocol III) was run on an 8% gel in lane 1, and the molecular weight standard (MWS) (Bio-Rad Broad Range) was run in lane # 3. Lane 2 was left blank. (b) Pool I (DE Sepharose-Protocol III) was run on a 12% gel with the molecular weight labeled beside each gel.

Fractions were assayed for antibacterial activity using the microdilution growth assay and antibacterial activity was present in both pre-gradient and post-gradient peak fractions. The peak fractions were further analyzed for protein composition using SDS-PAGE analysis (Figure 15).

Active fractions were pooled, Pool I (fractions 5-11), Pool II (fractions 19-39), Pool III (57-67) and Pool IV (89-105), concentrated by lyophilization, and following acid-urea gel

electrophoresis, were analyzed for antibacterial activity by applying to a lawn of *P. citreus* (acid-urea overlay). Distinct zones of inhibition were seen in the seeded lawn for pools I, II and IV, although growth of *P. citreus* was sparse. Zones of growth inhibition are shown in Figure 16. A tandem gel run simultaneously as the gel used to overlay the seeded agar was stained with coomassie blue (Figure 16a) to show position of protein bands occurring on the gel. LC-MS/MS analysis could not be performed on these samples because of contamination of the pools resulting from loss of refrigeration upon storage.

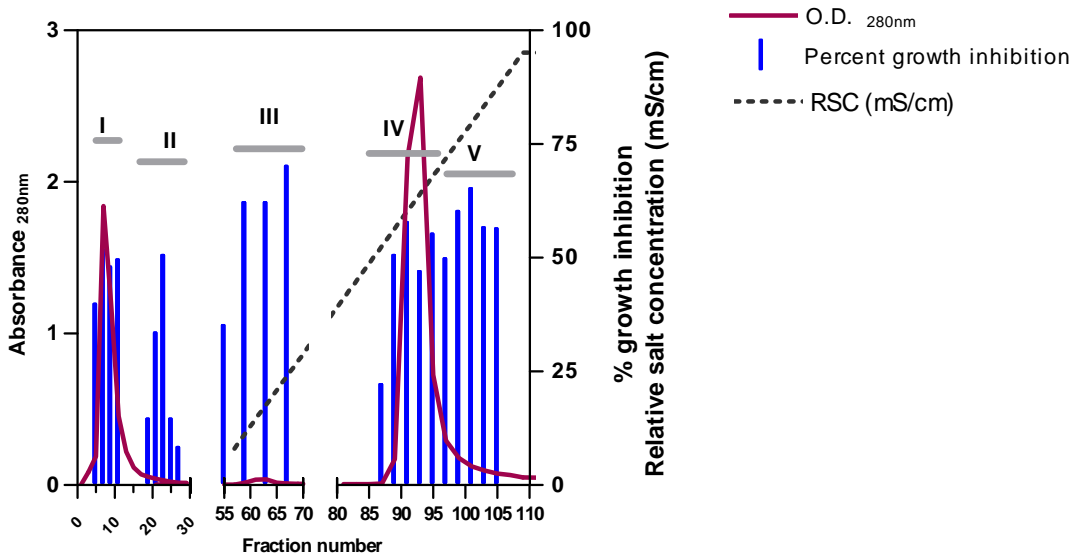


Figure 14 Protocol IIC-1: Fractionation of lysate on DE Sepharose column. Concentrated crude lysate (0.4 ml) mixed with equilibration buffer (0.1 ml) was applied to DE Sepharose column (1.0 x 4.5 cm) equilibrated with 0.02M Tris-HCl, 0.05M NaCl, pH 7.5. Fractions (1 ml) were collected at a flow rate of 1 ml/min at 25°C. Bound proteins were eluted from the column with a linear NaCl gradient, up to 0.9M NaCl. Antibacterial activity (blue bars) was measured by microdilution growth assays for all fractions tested and recorded as percent growth inhibition (for details see Methods).

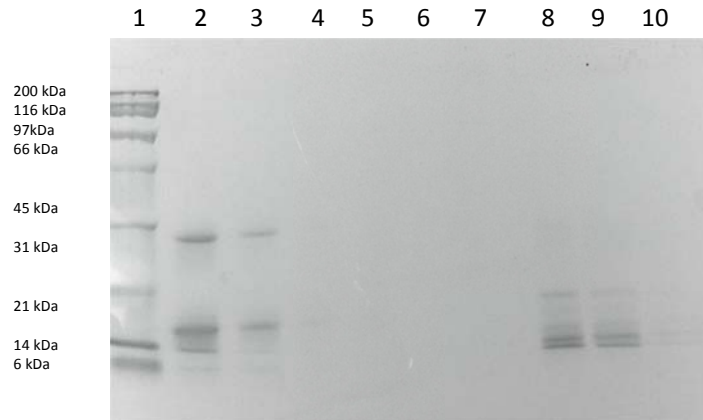


Figure 15 SDS-PAGE analysis of fractions from DE Sepharose column (Protocol IIC-1). Peak fractions were analyzed by SDS-PAGE. Molecular weight standard (MWS) (Bio-Rad Broad Range) was run in lane # 1. Lanes 2 to 10 contained fractions 7,9,11,13,63,65,91,93,95 respectively.

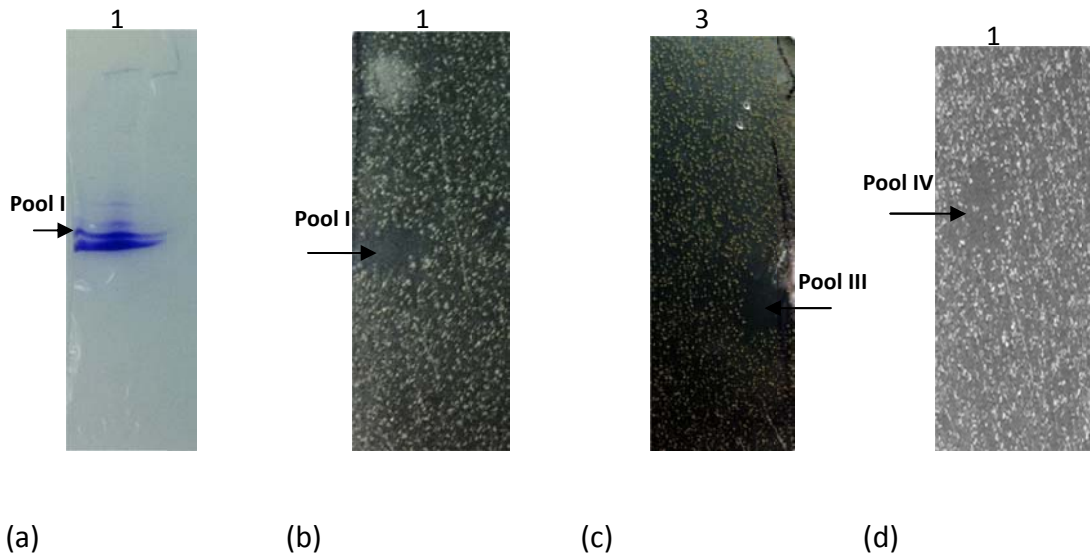


Figure 16 Acid-Urea PAGE analysis of concentrated pools of DE Sepharose (Protocol IIC-1). (a) Coomassie blue stained tandem run gel showing protein composition of the sample. All samples were electrophoresed on pre-run gels, washed in 10mM phosphate buffer pH 7.5 and overlaid on the agar seeded with *Planococcus citreus* and incubated 3 h at 30°C. Gel was removed and SCM poured on top and allowed to solidify. Plates were incubated overnight at 30°C. Pools I (b), Pool III (c) and Pool IV (d), showed zones of inhibition. Zone of inhibition of Pool I corresponded to protein band seen on coomassie blue stained tandem gel. Pool II and IV (not shown) showed no zones of inhibition.

The zone of inhibition seen with Pool I on the acid urea gel (Figure 16b) correlated to protein bands seen on coomassie blue stained tandem gel. Pool II and IV (not shown) showed no zones of inhibition. Pool IV (89-105) from DE Sepharose (Protocol IV) was

concentrated fifty-fold by lyophilization and reconstituted in ddH₂O. This sample was applied to a second DE Sepharose column run under similar conditions. Antibacterial activity was recovered post-gradient, with an increasing NaCl gradient (Figure 17). Although there is antibacterial activity, it is at a low level (below 20%), so it is difficult to distinguish the actual level of activity with these fractions that elute with high salt concentration. Peak activity fractions were pooled, lyophilized and reconstituted in ddH₂O and analyzed by SDS-PAGE and acid-urea gel electrophoresis. The lawn of target organism showed no zones of growth inhibition within the area subjected to acid-urea gel overlay, although a zone of inhibition was noted around the control antimicrobial peptide, HNP1 (a β -defensin).

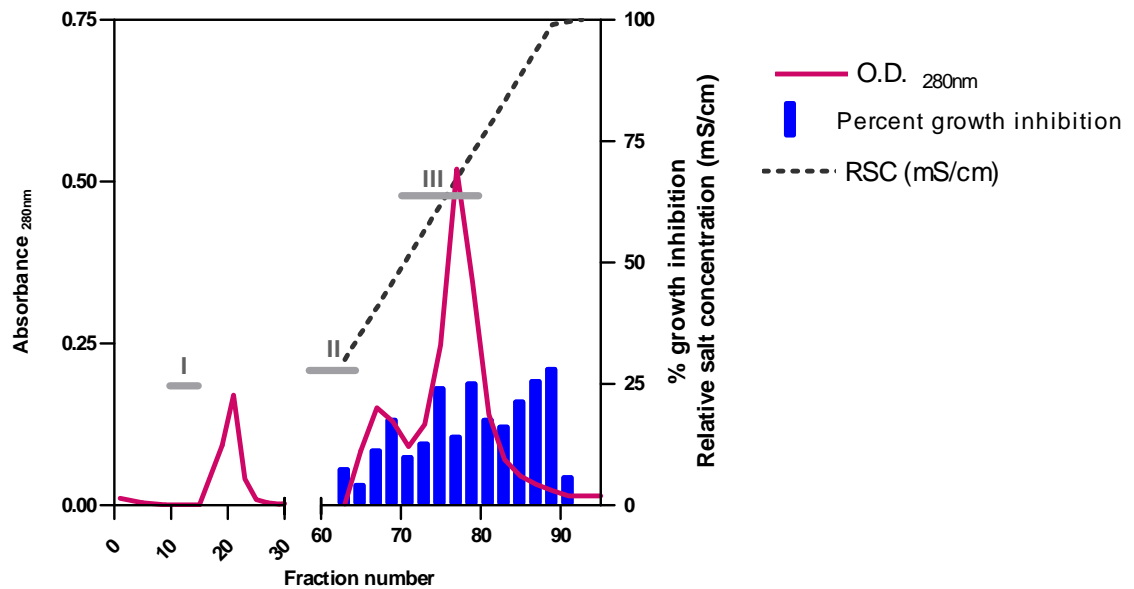


Figure 17 Protocol IIC-1: Fractionation of Pool IV from first DE Sepharose column. Pool IV (0.5 ml) mixed with an equal volume of equilibration buffer was applied to a second DE Sepharose column (2.5 x 4.5 cm) equilibrated with 0.02M Tris-HCl, 0.05M NaCl, pH 7.5. Fractions (1 ml) were collected at a flow rate of 1 ml/min at 25°C. Bound proteins were eluted from the column with a linear NaCl gradient, up to 0.9M NaCl. Antibacterial activity (blue bars) was measured by microdilution growth assay for all fractions tested and recorded as percent growth inhibition (for details see Methods).

Protocol IIC-2: DE Sepharose-sonicated lysate III

To improve separation and recovery of antibacterial activity the protocol for ion exchange chromatography was modified by the application of a step-wise elution gradient. A leukocyte lysate concentrated five-fold by lyophilization and reconstituted in 1 ml ddH₂O was applied to a DE Sepharose column and eluted with a two step linear NaCl gradient (Gradient 1-0.05M NaCl to 0.5M NaCl; Gradient 2- 0.5M NaCl to 1.0M NaCl). The chromatographic profile (Figure 18) shows four protein peaks, a pre-gradient and three post-gradient peaks. Fractions were assayed for antibacterial activity using the agar diffusion assay and antibacterial activity was present in the pre-gradient and post-gradient peak fractions. The fractions showing high antibacterial activity were further analyzed for protein composition by SDS-PAGE (Figure 19).

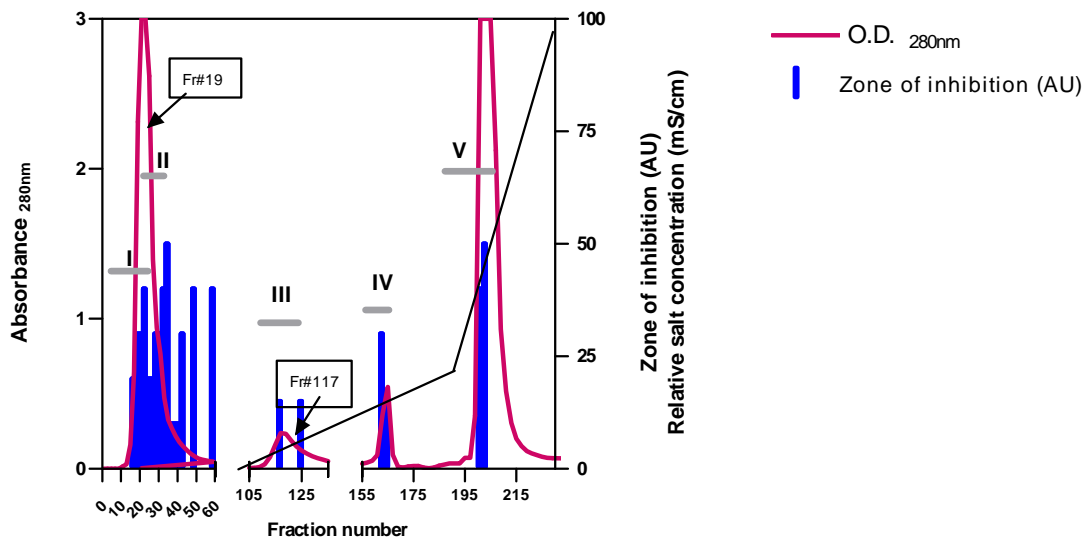


Figure 18 Protocol IIC-2: Fractionation of lysate on DE Sepharose column. Concentrated crude lysate (0.7 ml) mixed with equilibration buffer (0.3 ml) was applied to DE Sepharose column (2.5 x 4.5 cm) equilibrated with 0.02M Tris-HCl, 0.05M NaCl, pH 7.5. Fractions (1 ml) were collected at a flow rate of 1 ml/min at 25°C. Bound proteins were eluted from the column with a step-wise NaCl gradient (Gradient 1-0.5M NaCl; Gradient 2- 1.0M NaCl). Antibacterial activity (blue bars) was measured by agar diffusion assay for all fractions tested and recorded as activity units (for details see Methods).

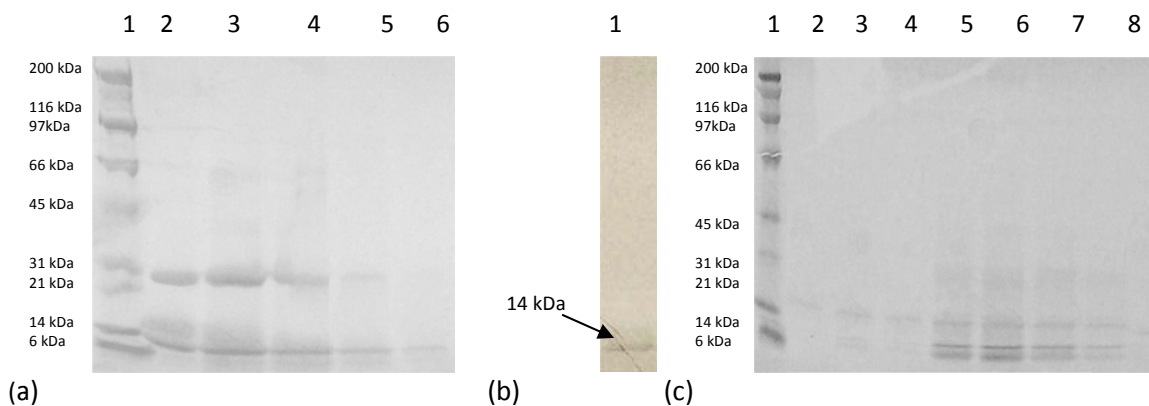


Figure 19 SDS-PAGE analysis of fractions from DE Sepharose column (Protocol IIC-2). Peak fractions were analyzed by SDS-PAGE. For each gel molecular weight standard (MWS) (Bio-Rad Broad Range) was run in lane # 1. (a) Lanes 2 to 6 contained fractions 17,19, 21 ,23,25 (of Pool I), respectively. (b) Lane 1 contained fraction 117 (of Pool III). (c) Lanes 2 to 8 contained fractions 163,165 (of Pool IV),199, 201, 203, 205, 207 (of Pool V), respectively.

Fractions 19 and 117 were also subjected to AU gel electrophoresis, and then applied to a lawn of target organisms for antibacterial activity. For both fractions, distinct zones of inhibition were seen in the seeded lawn (Figure 20).

Peak fractions were pooled, Pool I (16-24), lyophilized, reconstituted in ddH₂O and subjected to acid-urea electrophoresis and analyzed for antibacterial activity by overlay on seeded agar (Figure 21a). A tandem gel run under the same conditions as the gel used to overlay the seeded agar was stained with coomassie blue (Figure 21b). The protein bands correlated with the position of growth inhibition zones noted in the agar overlay (Figure 21a and b).

Pool III (115-121), which included active fraction 117, was chosen to be subjected to LC-MS/MS analysis. The excised band was sent to the University of Florida Protein Core and yielded two unique fragments with a total of 28 residues with sequence homology to Chain A of Hen egg white lysozyme, HEWL (see Table 6).

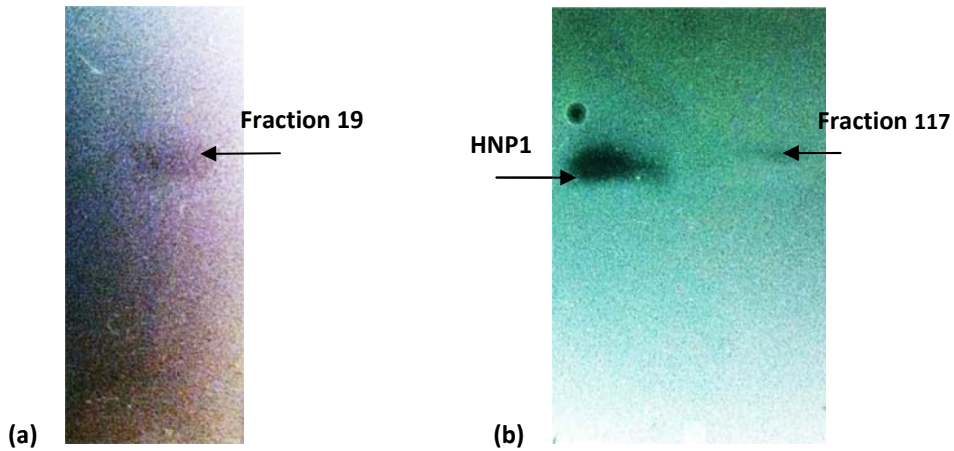


Figure 20 Acid-Urea Gel analysis of fractions from DE Sepharose (Protocol IIC-2). Overlay showing zone of inhibition with fractions from lysate from DE Sepharose. (a) Fraction 19 (lane 1), (b) HNP1 (25 µg/ml) lane 1, Fraction 117 (lane 2), were loaded onto pre-run gel and once electrophoresed, were washed in 10mM phosphate buffer pH 7.5 and overlaid on the agar seeded with *Planococcus citreus* and incubated 3 h at 30°C. Gel was removed and SCM poured on top and allowed to solidify. Plates were incubated overnight at 30°C.

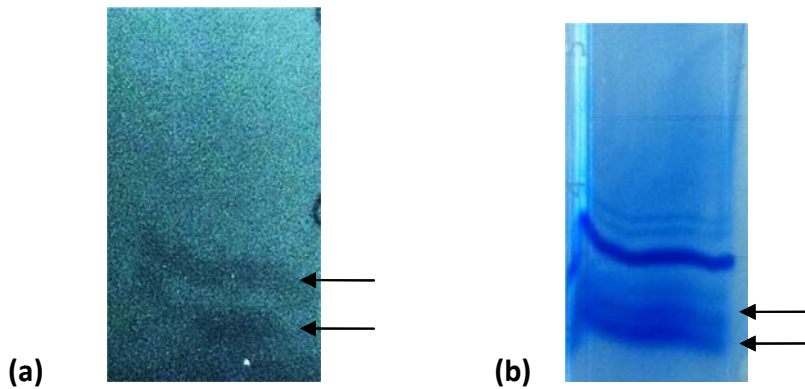


Figure 21 Acid-Urea Gel analysis of Pool I (Protocol IIC-2). Overlay showing zone of inhibition with concentrated pools from DE Sepharose Pool I. (a) Pool I (lane 1), was loaded onto pre-run gel and once electrophoresed, gels were washed in 10mM phosphate buffer pH 7.5 and overlaid on the agar seeded with *Planococcus citreus* and incubated 3 h at 30°C. Gel was removed and SCM poured on top and allowed to solidify. Plates were incubated overnight at 30°C. (b) Acid-urea tandem gel showing band corresponding to zone of inhibition. Tandem gel was run simultaneously as gel used for overlay and directly stained in coomassie brilliant blue R-250 for 1h and destained overnight.

Table 6 Internal Amino Acid Sequence Analysis of Proteins Isolated from Shark Leukocyte Lysates Analyzed by LC-MS/MS

Sample	Band	Sequence	ID
Pool I-DE Sepharose (Protocol IIB)	Band 1	TITLQVEPSDTIENVK IQDKEGIPPDQQR KSTLHLVLR	Polyubiquitin
Pool I-DE Sepharose (Protocol IIB)	Band 1	LIFAGK	Ubiquitin
Pool III-DE Sepharose (Protocol IIC-2)	Band 1	FESNFNTQATNR NTDGSTDYGILQINSR	Chain A, HEWL

Western Blot Analysis

Western blot analysis of pools was performed to determine whether histones and lysozyme could be detected using heterologous antisera such as rabbit polyclonal antibodies to human histones H2 and H4 and antibodies to hen egg white lysozyme (HEWL) as the primary antibodies. Goat anti-rabbit IgG was the secondary antibody used. No reactivity with any shark protein was noted with anti-histone antibodies indicating (a) lack of reactivity with the heterologous antibody or absence of histone fragment from lysates prepared by sonication. However, when primary antibody to HEWL was used against the same pool, reactivity was seen with a protein band in shark lysate that corresponds to the HEWL reactive control band (intensely stained in Figure 22), strongly suggesting the presence of lysozyme in shark leukocyte lysates.

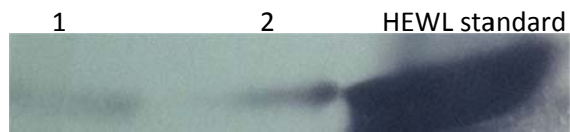


Figure 22 Western blot analysis. Western blot of fractionated lysate sample (Pool I, Protocol IIB) using rabbit anti-HEWL antibodies as the primary antibody. Sample was electrophoresed under non-reducing conditions. The blot was developed using goat anti-rabbit HEWL antibody. Lanes 1 and 2 are duplicate samples.

V. DISCUSSION

An integral function of innate immunity in all vertebrates is to protect the host against microbial invasion. Protection is achieved, in part, by the antimicrobial action of a variety of proteins (including enzymes) and peptides associated with body fluids (e.g., tears, saliva, mucus) and specialized cells in peripheral blood and tissues (e.g., cells lining the gut wall). While much is known of the antimicrobial proteins/peptides of mammals, amphibians and bony fish, relatively little information is available on antimicrobials of sharks particularly those associated with peripheral blood leukocytes. The specific aim of this study was to characterize antimicrobial proteins/peptides associated with shark blood leukocytes.

The composition of nurse shark peripheral blood differs significantly from that of teleosts (bony fish) and other vertebrates, particularly, humans. It is important to note that shark plasma contains 0.25M NaCl and 0.35M urea. The high concentration of urea in blood allows these animals to stay in osmotic balance. The high concentration of salt and urea in circulating blood has to be taken into account when developing protocols for processing serum/plasma and/or blood cells. Consequently, methods routinely used in human or mouse research have to be modified by extensive trial and error work on the bench. Staining of peripheral blood smears to obtain differential counts, poses a challenge since most staining reactions depend on acidic and basic reactions of cells. Furthermore, shark erythrocytes are nucleated (unlike that of humans), thus when blood cells undergo lysis (induced or otherwise) considerable quantities of nucleic acid

released from erythrocytes is present in cell lysates. The presence of nucleic acid can be a hindrance when attempting to isolate proteins/peptides from leukocyte lysates.

Several cell types found in shark peripheral blood are morphologically similar to mammalian cells, although corresponding functional definition for all types remains undetermined. Two types of granulocytic leukocytes are seen, one with deeply staining prominent eosinophilic granules similar in morphology to the mammalian eosinophil, the other with finer neutrophilic granules along with a bi- or tri-lobed nucleus. Ultra-structural studies have shown the shark eosinophil to contain crystalline material within the granules while the shark neutrophil granules are fibrous in nature (Stokes et al. 1971; Hyder et al. 1983; Smith et al. 1989). The proportion of eosinophilic leukocytes in shark blood is considerably higher (11.5%) than that seen in human peripheral blood (2.7%). This, in part can be an indication of a cellular immune response to parasites which are harbored by sharks (Borucinska et al. 1993). Functional studies have shown the shark neutrophil and monocyte-macrophage to be phagocytic, similar to their mammalian counterparts (Hyder et al. 1983; Smith et al. 1989). These two cell types have also been shown to chemotactically (i.e., directional migration) respond to appropriate stimuli (Obenauf et al. 1985; Smith et al. 1989). Some studies have shown in certain shark species, the shark thrombocyte to also be phagocytic (Yokoyama 1960; Fange 1968; Stokes et al. 1971; Ferguson 1976). The spindle shaped thrombocyte is a cell type not found in mammalian blood, and thrombocytes may have an important role in coagulation corresponding to the role of mammalian platelets (Gardner et al. 1969; Stokes et al. 1971; Wardle 1971). In addition to the mononuclear lymphocytes seen in

peripheral blood, immature, undifferentiated blast-like cells are also occasionally seen. The presence of blast-like cells in peripheral blood is not surprising since sharks with their cartilaginous skeleton do not have bone marrow, the site in mammals where leukocyte maturation and differentiation occurs. In sharks, the site of hematopoiesis (i.e., formation of erythrocytes and leukocytes) is the epigonal organ (a part of its reproductive system), a lymphoid organ, and the spleen (Fange 1977; Fange et al. 1981; Zapata 1981). Since cell lysates prepared from shark peripheral blood leukocytes show significant antibacterial activity, it is reasonable to assume that the factors responsible for this activity are most likely associated with the shark neutrophil and/or eosinophil as well as the monocyte-macrophage, an active phagocytic cell.

A variety of antimicrobial factors, include enzymes such as lysozyme (Petit et al. 1963; Jolles 1967), cathepsin G (Senior et al. 1982; Spitznagel 1990), phospholipase A2 (Dennis 1997), transferrin (Putnam 1975), and antibacterial proteins/peptides (e.g. defensins, magainins, cecropins and cathelicidins) (Boman 1991). As stated above these factors can be found in body fluids (e.g., saliva), mucus produced by the skin (e.g., frogs) and contained within intracellular cytoplasmic granules.

In mammalian granulocytic cells, antibacterial proteins/peptides and enzymes (e.g., lysozyme) are normally found sequestered within intra-cellular granules. My study has shown that cell lysates prepared from nurse shark peripheral blood leukocytes are antibacterial and inhibit the growth of *Planococcus citreus*, a salt tolerant bacterium. Since shark leukocyte lysates also contained lysozyme activity, it is likely that lysozyme is sequestered within intra-cytoplasmic inclusions (lysosomes) of one or more cell type,

most probably the phagocytic leukocytes. Lysozyme activity was detected in several lysate preparations and was distinct from the non-lysozyme antibacterial activity, the isolation and characterization of which was the focus of this study. Initial screening of lysates for lysozyme was accomplished using lysoplates (Osserman et al. 1966; Manchenko 1994) and enzyme activity was estimated from the size of the zones of lysis (clearing) of *Micrococcus lysodeikticus*. Chromatographic fractionation of crude lysates revealed lysozyme activity to be present in early and late fractions recovered from columns suggesting there might be two lysozyme molecules differing perhaps in one or more amino acid residues. Slight differences in primary structure can result in difference in over-all protein charge which would account for the difference in binding strength to the column. A protein corresponding to lysozyme was detected by Western blotting in active fractions. The presence of shark lysozyme in lysate was confirmed by amino acid sequence analysis of a 14 kDa protein separated by SDS-PAGE. The presence of multiple isoforms of lysozyme from the same organism or even the same biological sample has been shown to be the case in the skin mucosa of rainbow trout (Fernandes et al. 2004), the mantle of the pacific oyster (Itoh et al. 2010), and several other invertebrate species (Cancado et al. 2008; Ursic-Bedoya et al. 2008).

A microdilution growth assay was developed in order to achieve greater sensitivity and a method for quantifying the level of antibacterial activity which was expressed as percent inhibition of normal uninhibited bacterial growth. The assay, once standardized, was able to detect levels of antibacterial activity as low as 5% inhibition in titrated crude lysates. This assay proved to be reliable and reproducible and worked

over a fairly broad range of pH and salinity. The density of the target organism did, however, affect the detection sensitivity of the assay. With high bacterial densities the antibacterial activity of lysates was masked since growth inhibition could not be detected spectrophotometrically. A target cell density of 2.5×10^4 cfu/ml was determined to be optimal. *Planococcus citreus*, the target organism routinely used, grows over a relatively wide temperature range, 27° to 37 °C, with an optimal temperature of 30°C. The microdilution growth assay is now available and optimized for future studies.

The protein concentration of crude lysates, prepared by sonication, ranged from 2 to 8 mg/ml depending on the quantity of leukocytes (8×10^8 - 1×10^9 cells) harvested from whole blood. The level of antibacterial activity of crude lysates correlated to the protein concentration of the preparation. Although all lysates prepared showed antibacterial activity, when titrated the level was low for some samples (activity was undetectable following a 2-fold dilution) while in others activity was 16-fold or higher. Individual variation from animal to animal could account for the variation in activity level. Variation could be as a result of the time or temperature that the blood was collected, or the age or gender of the shark, or even the size of the shark. As blood collection was subject to all these conditions, it is difficult to ascribe a pattern or even control for these variations, given that several different sharks were used in the course of this study.

Initially, crude lysates were used as starting material for chromatographic separation of antibacterial activity. However, the recovery of activity in the active fractions was low. To improve recovery and increase yield crude lysates were subjected to solid phase

extraction as an initial first step in the isolation protocol. While activity was concentrated by this procedure it was present in a fraction that also contained the bulk of cellular protein and thus did not offer a significant advantage over lyophilization, a less harsh method to obtain concentrated starting material. Ion exchange chromatography of crude lysates concentrated by lyophilization resulted in recovery of significant yield (measured in activity units). Anion exchange chromatography of lysates was effective in separating lysozyme from non-lysozyme antibacterial activity. Initial fractionation of leukocyte lysates yielded several peptides in column fractions which were visualized by SDS-PAGE analysis and identified by N-terminal amino acid sequence analysis to have sequence homology to histone fragments. A variety of histone fragments have been shown to be antibacterial in other vertebrate species (Hiemstra et al. 1993; Robinette et al. 1998; Agerberth et al. 2000; Kawasaki et al. 2003; Patat et al. 2004; Silphaduang et al. 2006).

Several investigators have used more sophisticated methods of chromatography, such as HPLC and reverse phase HPLC, to isolate and purify antimicrobial proteins/peptides (AMPs) from biological samples; however, I found that employing a combination of one or more low pressure chromatography steps is sufficient to isolate the antibacterial activity from the crude cell lysates, and to further separate the active protein(s) from lysozyme, also present in lysates. Although the active protein/peptide(s) were not purified to homogeneity using this simple protocol, it was sufficient to separate the bulk of non-antibacterial material contained in lysates, and to yield a relatively “clean” preparation that contained two to four proteins (seen as distinct

bands) that could be easily distinguished on coomassie blue stained gels after SDS-PAGE analysis. Furthermore, the antibacterial activity of each protein could be visualized by zones of inhibition on bacterial lawns following transfer of proteins separated by acid urea gel analysis. Consequently, the position of growth inhibition zones on the bacterial lawn could be correlated to individual protein bands present on tandem run gels.

No immunoreactivity was obtained using antibodies to human histones. Therefore, the presence of histones could not be confirmed since no reactivity was noted on the blots. The lack of reaction could be due to (1) the antibodies not recognizing epitopes in nurse shark histone fragments or (2) the concentration of primary and secondary antibodies was not sufficient to get a reaction.

The presence of histone fragments isolated from peripheral blood leukocytes of the nurse shark exhibiting antimicrobial activity has never been shown. These histone fragments, identified by N-terminal sequencing as histones H2A, H2B and H4 (identified in a previous study (Hinds et al. 2001)) have been shown in the literature to be antimicrobial in nature (as mentioned above) and isolated from a wide range of organisms, especially aquatic organisms such as teleosts (Park et al. 1998; Birkemo et al. 2003; Fernandes et al. 2004; De Zoysa et al. 2009), bivalves (Li et al. 2007) and shrimp (Patat et al. 2004). Table 7 lists the histone fragments that have been identified by N-terminal amino acid sequence analysis in shark leukocyte lysates and their homology to other known antibacterial peptides identified in other species.

Table 7 Alignment of Amino Acid Sequence of Shark Histone Fragments

Histone Fragment	Source	Sequence	Reference
H2A	Nurse Shark H2A-1 Nurse Shark H2A-2 Toad stomach tissue (Buforin) Catfish (Parasin I) Pacific white shrimp hemocyte Rainbow trout skin secretions	SRSERAGLQFQK SIAISRSYRAGLQF AGRQKQGGKVRAKAKTRSSRAGLQFPVG// KGRGKQGGKVRAKAKTRSS SGRGKGGKVKGSSRSSRAGLQFPVGR// AERVGAGAPVYL	Present study Present study (Fernandes et al. 2002) (Park et al. 1996) (Park et al. 1998) (Patat et al. 2004)
H2B	Nurse Shark H2B-1 American oyster Nurse Shark H2B-2 Human Channel catfish Atlantic cod mucus Pacific white shrimp hemocyte	ESYSIYIK ESYAIYIK KGAK KRSRKEXY KAQKKDSKKR KRSRKESYSVYVYK// PDKAKTAPKKGSKKAVTKXA PEVAKPAAKKGSKKAVSKVA// AKHAVSEGTKAVT	Present study (Robinette et al. 1998) Present study (Frohm et al. 1996) (Bergsson et al. 2005) (Patat et al. 2004) (Seo et al. 2010)
H4	Nurse Shark H4-1 Fruit fly* Pacific white shrimp hemocyte Nurse Shark H4- 2 Fruit fly *	DNIQLITKPAIRQLARR KVLRDNIQGITKPAIRRLARRGGVK// RDNIQGITKPAIRRL RQGRTLYGFX LKRQGRTLYGFGG	Present study Present study

Note: Sequences are provided when available with references and sources with asterisk (*) are from deduced amino acid sequences not yet proven to be antibacterial, and obtained through BLAST searches.

Histones have long been known to have antibacterial activity (Hirsch 1958), however, it was not known how they would interact with bacteria in the event of an infection. It was almost 40 years later that Frohm et al (1996) suggested that histones were responsible for wound healing when they isolated histone fragments from human wound and blister fluid. Since then it has become clear that histones or histone fragments are not only present in the nucleus of various organisms, but also in the extra-nuclear region where they can be exposed to intracellular pathogens. The

discovery of neutrophil extracellular traps (NETs) (Brinkmann et al. 2004) has added to this theory of extra-nuclear histones, even expanding to the possibility that histones work in conjunction with these nets together to trap and kill invading pathogens (Brinkmann et al. 2004; Papayannopoulos et al. 2009). Histones because of their DNA and RNA binding capabilities may work differently in killing bacteria than other traditional antimicrobial peptides. Buforin II, a histone H2A fragment, has been shown to penetrate through the cell membrane and to accumulate inside bacterial cell, leading to the theory that it may inhibit cellular function by binding to nucleic acid (Park et al. 2000). It is therefore not outside the realm of possibilities that the nurse shark H2A fragment which has high homology to buforin, could work in a similar manner. Histone fragments were not identified in shark lysates prepared by sonication indicating that the method of choice for preparing lysates does appear to determine the composition of cellular proteins that will be recovered including those that are antibacterial.

Amino acid sequence analysis also generated some unexpected findings. Two very similar hemoglobin fragments, as well as the protein ubiquitin, were isolated from active chromatographic pools. These two protein fragments are known for having other functions. Hemoglobin is a protein that transports oxygen (Altman et al. 1961) in erythrocytes. Ubiquitin is a protein found everywhere in the cell, thus its name. Ubiquitin's main function is to covalently bind to proteins to act as a tag, and to shuttle the tagged protein to the proteasome for degradation, thereby playing a role in MHC Class I antigen presentation, as well as in vertebrate immune response via cytotoxic T cells (Kindt et al. 2007). Hemoglobin from various species, in its intact form, as well as

fragmented, (Parish et al. 2001; Nakajima et al. 2003) has been shown to be antibacterial. Ubiquitin, on its own has not been shown to be antibacterial. However, it has been associated, i.e. covalently bound, to histones (e.g. histone H1), which itself was antibacterial (Wang et al. 2002). In addition, ubiquicidin, a protein with high homology to a precursor of ubiquitin, has also been shown to be antibacterial (Hiemstra et al. 1999).

This study has revealed that shark leukocyte lysates contain several antibacterial proteins/peptides, several of which show homology to antibacterial proteins described for species of different phyla, indicating conservation of form and function through evolution. This premise is particularly true for a protein such as lysozyme. Sequence analysis also identified proteins with no known homology and which could very well be unique to the shark. The elucidation of the mechanism of antibacterial activity of lysate proteins/peptides was beyond the scope of this study, and remains a future goal. It is clear from the data presented that several antibacterial proteins/peptides associated with non-oxidative antibacterial activity of mammalian leukocytes are also found in the nurse shark, a primitive vertebrate. With this theory in mind, it could be said that shark antibacterial proteins/peptides, play a significant role in the shark's resilience to infection and disease and its longevity.

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APPENDIX I

1.0 Preparation of Buffers, Stock Solutions and Media

Buffers and stock solutions were filtered through a 0.45 micron Nalgene filter before use. Unless otherwise stated, all sterilization of liquids and culture media was carried out in an autoclave at 121°C for 20 min under 15 lbs. pressure. Autoclaved material was cooled to room temperature before storage at 4°C or stored at room temperature (RT).

Solutions were prepared as follows:

0.5 M sodium phosphate ($\text{Na H}_2\text{PO}_4$)-acidic solution:

- 34.5g $\text{Na H}_2\text{PO}_4$, dry weight (*Sigma, MO*) was added to sterile deionized distilled (dd) water (H_2O) and the volume brought up to 500 ml.

0.5 M sodium phosphate (Na_2HPO_4)-basic solution:

- 35.5g Na_2HPO_4 , dry weight (*Sigma, MO*) was added to sterile dd H_2O and the volume brought up to 500 ml until salt completely dissolved.

Stock 0.5 M sodium phosphate (PB), pH 7.5

- 0.5 M sodium phosphate buffer of pH 7.5 was prepared by mixing appropriate volumes of 0.5 M acid and base sodium phosphate stock solutions. To attain a pH of 7.5, 95 ml of the acid was added to approximately 500 ml of the base.

Phosphate buffered saline (0.005M NaPO_4 , 0.14 M NaCl, pH 7.3)

- 2 g NaCl (*Fisher Scientific, GA*) was added to 0.005M PB (diluted 1:100 from stock), and the mixture stirred until completely dissolved. The pH was adjusted

to 7.3 with 1N HCl (approximately 5 drops). The final volume was adjusted to 250 ml with the diluted PB. The buffer was then sterilized.

Shark phosphate buffered saline (0.05 M NaPO₄, 0.35M urea, 0.2 M NaCl, pH 7.3)

- 1.17 g NaCl (*Fisher Scientific, GA*) and 2.1g urea (*Fisher Scientific, GA*) was added to 0.5 M PB (diluted 1:10 from stock) and the mixture stirred until completely dissolved. The pH was adjusted to 7.3 with 1N HCl and the final volume was adjusted to 100 ml with the diluted PB before sterilization.

Shark RPMI medium (RPMI 1640 containing 0.35M urea, 0.2 M NaCl)

- This isotonic medium for shark blood cells was prepared by dissolving 3.5 g NaCl and 6.3g urea (*Fisher Scientific, GA*) in 300 ml RPMI 1640 medium (*Gibco/Invitrogen, CA*), containing 0.025 M HEPES Buffer with L-glutamine, 0.1 micron filtered, pH 7.3. The medium was further sterilized by filtration through 0.45 micron Nalgene filter and stored at 4°C.

0.25M NaCl solution

- An isotonic salt solution was used for suspending shark leukocytes and was prepared by dissolving 3.65 g of NaCl in 250 ml of ddH₂O and sterilized.

3.2% NaCl solution

- This solution was used for washing salt tolerant bacterial stock culture and was prepared by dissolving 3.2 g of NaCl in 100 ml of ddH₂O and sterilizing.

0.2% NaCl solution

- This hypotonic solution was used to lyse contaminating erythrocytes present in shark leukocytes isolated from whole blood, and was prepared by dissolving 0.2 g of NaCl in 100 ml of ddH₂O and sterilizing.

2.5% NaCl solution

- This hypertonic solution was used to neutralize the hypotonic salt solution during lysis of RBC's in shark leukocyte suspensions and was prepared by dissolving 2.5 g of NaCl in 100 ml of ddH₂O and sterilizing.

0.06 M phosphate buffer (0.06M NaPO₄, variable pH)

- 60 ml 0.5 M phosphate stock buffer (PB) was diluted in 400 ml ddH₂O and then the pH was adjusted, using 1N NaOH/1N HCl .The volume was brought up to 500 ml before sterilization.

0.06 M phosphate buffer-NaCl (0.06M NaPO₄, 0.14M NaCl, variable pH)

- 0.818g NaCl was diluted in 80 ml of 0.06M PB and then the pH was adjusted using 1N NaOH/1N HCl .The volume was brought up to 100 ml before sterilization.

Artificial sea water salt solution 5x stock (2.585M NaCl, 0.125M MgSO₄, 0.125M MgCl₂,

0.05 M KCl, pH 7.5)

- 151.06g NaCl, 15.05g MgSO₄, 25.41g MgCl₂, and 3.73g KCl (*Sigma, MO*) were added to ddH₂O, and after the pH was adjusted to 7.5, using 1N NaOH/1N HCl,

the volume brought up to 1000 ml. The stock solution was diluted five-fold to obtain a 1x working solution used to prepare media.

2.0 Staining Solutions

Modified Coomassie stain (23.47% methanol, 13% formaldehyde), used for staining radial diffusion assay plates, was prepared by,

- 10 mg Coomassie Blue R-250 dye (*Sigma, MO*) was dissolved in 63 ml ddH₂O, 27 ml methanol (*Fisher Scientific, GA*) and 15 ml 37 % formaldehyde (*Sigma, MO*) to give a total volume of 115 ml. The solution was stored in a dark bottle at room temperature.

Modified Coomassie destain (10% acetic acid, 2% dimethylsulfonate-DMSO)

- 50 ml glacial acetic acid (*Fisher Scientific, GA*) and 10 ml DMSO (*Sigma, MO*) was added to 440 ml ddH₂O and stored at room temperature.

Laemmli Coomassie stain for staining SDS-PAGE gels.

- 1.875 g Coomassie Blue R-250 dye (*Sigma, MO*) was dissolved in a mixture of 227 ml methanol (*Fisher Scientific, GA*) and 94 ml glacial acetic acid (*Sigma, MO*) and the volume adjusted with 179 ml ddH₂O. The solution was stored at room temperature.

Modified Laemmli Coomassie destain for SDS-PAGE gels.

- 100 ml glacial acetic acid (*Fisher Scientific, GA*), 375 ml methanol (*Sigma, MO*) and 25 ml glycerol (*Sigma, MO*) was added to 500 ml ddH₂O for a total volume of 1000 ml and stored at room temperature.

Modified Coomassie stain for staining PVDF membranes.

- 0.02g Coomassie Blue R-250 dye (*Sigma, MO*) was dissolved in a mixture of 40 ml methanol (*Fisher Scientific, GA*) and 60 ml ddH₂O. The solution was stored at room temperature.

3.0 Electrophoresis Solutions and Buffers

Electrophoresis 10X stock buffer (Oxford formula)

- 30g Tris and 144g glycine (*Bio-Rad, CA*) was dissolved in 900 ml ddH₂O, the pH was adjusted to 8.8 with 1N NaOH/1N HCl and the total volume was adjusted to 1000 ml with ddH₂O. The solution was filter sterilized (0.4 micron), and stored at 4°C.

Electrophoresis 1X running buffer-SDS

- 10 ml 10% SDS (*Sigma, MO*) was added to 100 ml 10X Electrophoresis buffer diluted in 700 ml ddH₂O. The pH was adjusted to 8.8 with 1N NaOH/1N HCl and the total volume was brought up to 1000 ml.

Sample buffer for SDS-PAGE (5X non-reducing)

- 0.6 ml 1M Tris-HCl, pH 6.8, 5 ml 50% glycerol (*Sigma, MO*) in ddH₂O, 2 ml 10% SDS and 1 ml 1% bromophenol blue in ddH₂O was added to 1.4 ml ddH₂O, and filter sterilized (0.4 micron), and stored at -20°C.

Sample buffer for SDS-PAGE (5X reducing)

- 0.6 ml 1M Tris-HCl, pH 6.8, 5 ml 50% glycerol in ddH₂O, 2 ml 10% SDS, 0.5 ml 2-mercaptoethanol (*Sigma, MO*) and 1 ml 1% bromophenol blue (*Sigma, MO*) in ddH₂O was added to 0.9 ml ddH₂O, filter sterilized (0.4 micron), and stored at -20°C.

30% acrylamide and 0.8% bis-acrylamide

- 30 g acrylamide and 0.8 g bis-acrylamide (*Sigma, MO*) were very carefully added to 100 ml ddH₂O and mixed. The solution was stored in a dark bottle at 4 °C.

60% acrylamide and 1.6% bis-acrylamide

- 60 g acrylamide and 1.6 g bis-acrylamide (*Sigma, MO*) were very carefully added to 100 ml ddH₂O and mixed. The solution was stored in a dark bottle at 4 °C.

Resolving buffer- 1.5M Tris-HCl (pH 8.8)

- 75 ml 2 M Tris-HCl, pH 8.8 (*Fisher Scientific, GA*) and 4ml 10% SDS were added to 21 ml ddH₂O, and mixed. The solution was stored at room temperature.

Stacking buffer- 0.5 M Tris-HCl (pH 6.8)

- 50 ml 1M Tris-HCl (pH 6.8) and 4ml 10% SDS were added to 46 ml ddH₂O, and mixed. The solution was stored at room temperature

Acid-urea electrophoresis buffer-5% acetic acid

- 50 ml glacial acetic acid (*Sigma, MO*) was added to 950 ml ddH₂O. The solution was filter sterilized (0.4 micron) and stored at 4°C.

Acid-urea sample buffer-9M urea in 5% acetic acid

- 2.7 g urea was added to 2 ml ddH₂O and mixed until dissolved. The solution was then mixed with AG501-X8 resin (*Bio-Rad, CA*) to deionize for 20 min. The solution was decanted, 0.25 ml glacial acetic acid was added and the volume was adjusted to 5 ml final volume with ddH₂O. 0.1g methyl green was added to the final solution, which was filter sterilized (0.4 micron) and stored at -20°C.

Acid-urea gel wash buffer-0.01 M NaPO₄, pH 7.5

- 10 ml 0.5 M Phosphate stock buffer (PB) was diluted in 490 ml ddH₂O and then the pH was adjusted, using 1N NaOH/1N HCl. The buffer was then sterilized.

4.0 Chromatography Buffers

For ion exchange chromatography, the following buffers were employed:

Equilibration buffer: 0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5

- 1.57 g Tris-HCl was added to 400 ml ddH₂O and the pH was adjusted to 7.5 with 1N NaOH. The final volume was adjusted to 500 ml with ddH₂O and the buffer filter sterilized.

Elution buffer A: 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5

- 11.69g NaCl was added to 400 ml 0.02 M Tris-HCl, the pH adjusted to 7.5 with 1N NaOH and the final volume adjusted to 500 ml with ddH₂O before the buffer was filter sterilized.

Elution buffer B: 0.02 M Tris-HCl, 1.0M NaCl, pH 7.5

- 11.69g NaCl was added to 200 ml of 0.02 M Tris-HCl, the pH was adjusted to 7.5 with 1N NaOH and the final volume adjusted to 250 ml with ddH₂O before the buffer was filter sterilized.

For heparin affinity chromatography, the following buffers were employed:

Equilibration buffer: 0.1 M Tris, 0.01 M Citric acid, 0.075M NaCl, pH 7.5

- 12.11 g Trizma base (*Sigma, MO*), 1.47 g citric acid (*Fisher Scientific, GA*) and 2.191 g NaCl was added to 400 ml ddH₂O and the pH was adjusted to 7.5 with 1N NaOH. The final volume was adjusted to 500 ml with ddH₂O and the buffer filter sterilized.

Elution buffer: 0.1 M Tris, 0.01 M citric acid, 2 M NaCl, pH 7.5

- 12.11 g Trizma base (*Sigma, MO*), 1.47 g citric acid (*Fisher Scientific, GA*) and 58.44 g NaCl was added to 400 ml ddH₂O and the pH was adjusted to 7.5 with 1N NaOH. The final volume was adjusted to 500 ml with ddH₂O and the buffer filter sterilized.

5.0 Culture Media

Double-strength Artificial Sea Water Complete Broth (2x SCM), pH 7.5

- 5g peptone, 1g yeast extract, 3 ml glycerol, and 0.2g glycerol phosphate were added to 400 ml of single strength artificial sea water salt solution (see above), and the mixture stirred and heated until dissolved. The pH was adjusted to 7.5 with 1N NaOH and the volume made up to 500 ml. The broth was aliquoted (250 ml) to screw cap conical flasks, sterilized and stored at 4°C.

Artificial Sea Water Complete Broth (1x SCM), pH 7.5

- 5g peptone, 1g yeast extract (*Difco, MI*), 3 ml glycerol, and 0.2g glycerol phosphate (*Sigma, MO*) was dissolved in 1x artificial sea water salt solution (5 fold diluted stock) solution. The pH was adjusted to 7.5 with 10 N NaOH, and the volume brought up to 1000 ml with 1x artificial sea water. Aliquots (50 ml) were sterilized in 125 ml screw cap conical flasks and stored at 4°C.

1% agar underlay with 0.02% Tween 20 for agar diffusion assay

- 2 g dehydrated agarose (*Sigma, MO*) was dissolved in 200 ml of 0.005 M PBS, pH 7.5, by heating the mixture. 20 µl Tween 20 (*Bio-Rad, CA*) was added to the mixture and the agar sterilized, cooled to 44°C in a water bath and dispensed as 10 ml aliquots into sterile 20 ml screw cap tubes and stored at 25°C (room temperature).

1% agar overlay with SCM

- 2 g dehydrated agarose (*Sigma, MO*) was added to 2x artificial sea water complete broth, and the mixture heated to dissolve the agar which was sterilized and cooled to 44°C in a water bath. The agar medium was dispensed as 10 ml aliquots into sterile 20 ml screw cap tubes which were stored at 25°C (room temperature).

2% agarose in 0.06M PB, pH 6.2

- 4 g dehydrated agarose was dissolved in 200 ml of 0.06M PB, pH 6.2, by heating and sterilized before dispensing as aliquots (100 ml) to 125 ml screw cap conical flasks. Used in the preparation of lysoplates.

Tryptic Soy Broth

- 30 g dehydrated tryptic soy broth powder (*Difco, MI*) was suspended in 1 L ddH₂O and warmed slightly to dissolve. The broth was aliquoted (50 ml) to screw cap conical flasks, sterilized and stored at room temperature.

Tryptic Soy Agar

- 40 g dehydrated tryptic soy agar powder (*Difco, MI*) was suspended in 1 L ddH₂O and warmed slightly to dissolve. The broth was aliquoted (15 ml) to screw cap conical flasks, sterilized and stored as slants at room temperature.

6.0 Reagents

3.8% Trisodium citrate (anticoagulant)

- 1.9g trisodium citrate (*Sigma, MO*) was dissolved in 50 ml shark RPMI, pH 7.3-7.5, and filter sterilized with a 0.45 micron Nalgene filter.

Human Neutrophil Peptide 1, HNP1

- Lyophilized pellet (*Sigma, MO*) reconstituted to 25ug/ml in sterile ddH₂O. This served as an antibacterial control in antibacterial assays.

Chicken egg white lysozyme

- A 1 mg/ml stock solution of chicken egg white lysozyme (muramidase) EC 3.2.1.17 (*Sigma, MO*) was prepared in ddH₂O, and diluted 1:100 (final concentration: 0.01 mg/ml) to use as a positive control in lysozyme assay plates (lysoplates).

Cell viability test reagent

- An isotonic reagent for shark leukocytes consisting of 0.1% trypan blue in 0.25M NaCl was prepared by dissolving 0.05 g trypan blue stain in 50 ml 0.25M NaCl. The solution was filtered and stored in 1 ml aliquots at RT.

7.0 Polyacrylamide Gels

Native-PAGE resolving gel (7.5%)

- 2.5 ml 30% acrylamide:0.8% bis-acrylamide (*Sigma, MO*), 2.5 ml 1.5M Tris-HCl, pH 8.8 (*Fisher Scientific, GA*), 5 ml ddH₂O, 50 µl 10% ammonium persulfate (*Bio-*

Rad, CA) and 10 μ l TEMED (*Bio-Rad, CA*) were gently swirled together until mixed. This mixture was immediately pipetted into assembled gel cassettes (*Bio-Rad, CA*) and allowed to polymerize at room temperature for 1 h.

Native-PAGE resolving gel (10%)

- 3.33 ml 30% acrylamide:0.8% bis-acrylamide (*Sigma, MO*), 2.5 ml 1.5M Tris-HCl, pH 8.8 (*Fisher Scientific, GA*), 6.67 ml ddH₂O, 50 μ l 10% ammonium persulfate (*Bio-Rad, CA*) and 5 μ l TEMED (*Bio-Rad, CA*) were gently swirled together until mixed. This mixture was immediately pipetted into assembled gel cassettes (*Bio-Rad, CA*) and allowed to polymerize at room temperature for 1 h.

Native-PAGE stacking gel (5%)

- 0.67 ml 30% acrylamide:0.8% bis-acrylamide (*Sigma, MO*), 1 ml 0.5 M Tris-HCl, pH 6.8 (*Fisher Scientific, GA*), 2.3 ml ddH₂O, 30 μ l 10% ammonium persulfate (*Bio-Rad, CA*) and 5 μ l TEMED (*Bio-Rad, CA*) were gently swirled together until mixed. This mixture was immediately pipetted into assembled gel cassettes (*Bio-Rad, CA*) on top of resolving gel and allowed to polymerize with appropriate sized combs at room temperature for 30 m. Polymerized gels were stored at 4 °C until ready to use.

8.0 Acid-Urea Gels

Acid-urea gel (12.5%)

- 4.8 g urea (*Fisher Scientific, GA*) was mixed with 7.8ml ddH₂O until dissolved, then 3.34 ml 60% acrylamide:1.6% bis-acrylamide (*Sigma, MO*), 0.87 ml glacial acetic acid (*Fisher Scientific, GA*), 0.30 ml 10% APS (*Bio-Rad, CA*) and 0.12 ml TEMED (*Bio-Rad, CA*) were added and gently swirled until mixed. This mixture was immediately pipetted into assembled gel cassettes (*Bio-Rad, CA*) and allowed to polymerize at 30°C with appropriate sized combs for up to 2 hours. Polymerized gels were stored at 4 °C until ready to use.

9.0 Target Bacteria for antibacterial assays

The following bacterial cultures were used as target organisms in antibacterial assays:

- *Planococcus citreus* (NCIMB 1493) was the target organism used in the antibacterial assays and was kindly provided by Dr. Valerie Smith, St. Andrews University, Scotland. The organism has been shown to be a problem in commercial shrimp culture implicated in shrimp spoilage (Alvarez et al. 1981).

10.0 Cell Walls for muramidase activity

The following cell wall preparation was used as target organism in lysozyme assays:

- A lyophilized suspension of cell walls of *Micrococcus lysodeikticus* (ATCC 4698) was used as the substrate for muramidase (lysozyme) activity in lysoplates.

11.0 Western Blot Buffers and Reagents

Transfer buffer

- 100 ml 10X Electrophoresis buffer was diluted in 700 ml ddH₂O. The pH was adjusted to 8.8 with 1N NaOH/1N HCl and the total volume was brought up to 900 ml.
- 100 ml methanol was added to the buffer (to facilitate transfer of smaller proteins) and mixed. The buffer was stored at 4 °C.

Blocking solution-5% nonfat dried milk

- A 5% nonfat dried milk solution was prepared by dissolving 50 g nonfat dried milk (*Carnation*) in 1 L ddH₂O. Fresh blocking solution was prepared each time.

Tris buffered saline (TBS)

- 11.69g NaCl was added to 400 ml 0.02 M Tris-HCl and the pH adjusted to 7.5 with 1N NaOH, and the final volume adjusted to 500 ml with ddH₂O before the buffer was filter sterilized.

Wash diluent buffer-1% milk in TBS with Tween 20

- 10 g nonfat dried milk was added to 1 L TBS and mixed until dissolved. 100 µl Tween 20 (*Bio-Rad, CA*) was added and solution stored at 4 °C.

Antibody buffer-1% milk in TBS

- 1 g nonfat dried milk was added to 100 ml TBS and mixed until dissolved and solution stored at 4 °C.

Rabbit anti-human H4 polyclonal antisera

- Affinity-purified rabbit anti-acetyl-histone H4 (*Sigma, MO*) antibody was diluted 1:750 in antibody buffer and stored at 4 °C.

Rabbit anti-human HDAC4 (H4) polyclonal antisera

- Affinity purified rabbit anti-histone deacetylase 4 (*Sigma, MO*) antibody was diluted 1:1000 in antibody buffer and stored at 4 °C.

Rabbit anti-human HDAC2 (H2) polyclonal antisera

- Affinity purified rabbit anti-histone deacetylase 2 (*Sigma, MO*) antibody was diluted 1:2000 in antibody buffer and stored at 4 °C.

Rabbit anti-hen egg white lysozyme polyclonal antisera

- Rabbit anti-HEWL (*Rockland, PA*) was diluted 1:1000 in antibody buffer and stored at 4 °C.

Peroxidase conjugated goat anti-rabbit IgG antisera

- Goat anti-rabbit IgG (*Rockland, PA*) was diluted 1:1000 in antibody buffer and stored at 4 °C.

Horseradish peroxidase (HRP) conjugate substrate kit

- The kit (*Bio-Rad, CA*) was assembled according to the manufacturer's instructions: 600 µl HRP color reagent B (containing hydrogen peroxide) was added to 100 ml of 1x HRP color development buffer at RT. This solution was added to 20 ml of HRP color reagent A (containing 4-chloro-1-naphthol in diethylene glycol) and reagent used immediately.

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PUBLICATIONS AND PRESENTATIONS

Hinds, N., Smith, S.L. (2001). *Antibacterial activity of shark leukocyte lysates*, Federation of American Societies for Experimental Biology Journal, 15(4).

Hinds, N., Smith, S.L. (2003). *Antibacterial activity of associated with histone fragments present in shark leukocyte lysates*, Paper presented at the 9th Congress of the International Society for Developmental and Comparative Immunology, St. Andrews, Scotland, UK.

Hinds, N., Smith, S.L. (2007). *Characterization of constitutive antimicrobial activity associated with leukocyte lysates from the nurse shark, *Ginglymostoma cirratum**, Poster presented at the Proceedings of the Annual American Society for Immunology, Miami, FL.