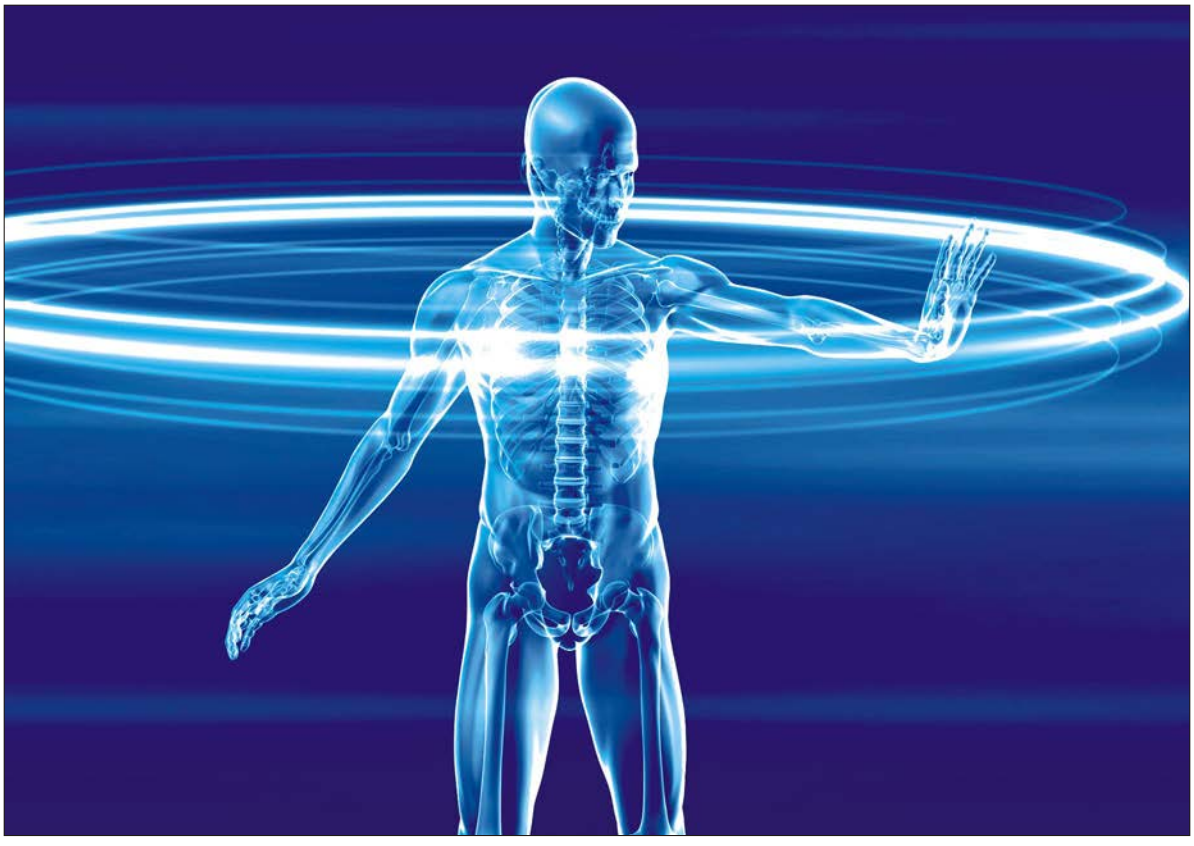

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Antimicrobial Peptides from the Marine Fishes

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Abstract: Fishes are one of the organisms that have managed to survive in a milieu of pathogenic organisms. The primary interference of fish with their environment happens through a mucous layer that covers its entire body. Marine fishes possess antimicrobial peptides as a part of their defense system, which are mainly present in the mucous layer indicating that they eliminate the pathogenic bacteria before they enter the skin barrier. A number of α-helical Antimicrobial Peptides (AMP) such as Pardaxins, Misgurin, Pleurocidins, Parasin, Oncorhyncin II and III, Chrysophsin and HFIAP (Hagfish Intestinal Antimicrobial Peptide) have been isolated from different species of fishes. However, studies on the role of antimicrobial peptides in fishes are very limit. Various mechanisms developed by multicellular organisms in nonspecific immunity raises questions on the role of antibiotic peptide as a deterrent against infection. The present study provides a general introduction to the subject with special emphasis on the role of bioactive peptides in marine fishes.

Key words: Marine fishes, antimicrobial peptide, mucous, immunity, bacteria

INTRODUCTION

Antimicrobial Peptides (AMPs) are regarded as an important component of the first-line defence in various animal species and are even more important in fish when compared with mammals as fish rely more on their innate immune system (Hancock, 1997; Hancock and Scott, 2000). In addition to the highly specific cell-mediated immune system, vertebrates and other organisms have a defense system made up of distinct groups of broad-spectrum antibacterial peptides (Boman, 1991, 1994, 1995; Zasloff, 1992). Rameshkumar *et al.* (2009a) proved that marine crabs *Charybdis lucifera* possess an antimicrobial peptide in their hemolymph. The main advantage of the antibacterial peptides as factors of innate immunity is that they can function without either high specificity or memory. An antibacterial peptide which is isolated from *Thalamita crenata* shows immense activity towards human bacterial pathogens (Rameshkumar *et al*., 2009b). Antibacterial peptides are promptly synthesized at low metabolic cost, easily stored in large amounts and readily available shortly after an infection. The haemolymph proteins of marine invertebrates are unique in composition, as they do not contain immunoglobulin or albumin like proteins and the protein (Rameshkumar *et al*., 2009c).

Several AMPs have been isolated from fish, such as Pleurocidins from winter flounder, *Pleuronectes americanus* (Walbaum), *American plaice, Hippoglossoides platessoides*

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(Fabricius) and Atlantic halibut, *Hippoglossus hippoglossus* (Cole *et al*., 1997; Douglas *et al*., 2001, 2003a,b). Misgurin from loach, *Misgurnus anguillicaudatus* (Cantor) (Park *et al*., 1997), Hagfish Intestinal Antimicrobial Peptides (HFIAPs) from hagfish, *Eptatretus burgeri* (Girard) (Hwang *et al*., 1999). Chrysophsins from red sea bream, Chrysophrys major (Iijima *et al*., 2003). Piscidin or Moronecidin from white bass, *Morone chrysops* (Rafinesque) and striped bass, *Morone saxatilis* (Walbaum) (Silphaduang and Noga, 2001; Lauth *et al*., 2002) and Hepcidins from several species of fish (Shike *et al*., 2002; Douglas *et al*., 2003a).

Antibacterial polypeptides belong to a larger group of naturally occurring short polypeptides, sharing similar amphipathic α-helical structures, which can interact strongly and permeate phospholipid membranes (Segrest *et al*., 1990; Saberwal and Nagaraj, 1994). Examples are bhemolysin from *Staphylococcus aureus*, the bee venom Melittin and the Moses sole fish cytotoxic peptide Pardaxin. In spite of their structural similarities to the antibacterial peptides they differ markedly in their functions. While antibacterial peptides have little or no cytolytic activity against normal mammalian cells at concentrations at which they inhibit the growth of bacteria, melittin is highly hemolytic and antibacterial (Boman *et al*., 1989) and 6-hemolysin is hemolytic but devoid of antibacterial activity (Dhople and Nagaraj, 1993).

Immune Mechanisms of Fish

Fish which form minor and major links in food webs of the aquatic ecosystems harbor a wide assay and a time large numbers of parasites. Fish represent not only the earliest, but also the largest class of vertebrates. This triumph of fish has been accomplished despite the fact that they possess both slower and less developed adaptive immune systems than these of higher vertebrates. Due to the aquatic environment, fish have unique anatomical and physical characteristics. Fish live in intimate contact with an environment containing both saprophytic and pathogenic microbes capable of digesting and degrading fish tissues (Ellis, 2001; Plouffe *et al*., 2005). The slow adaptive immune response of fish makes innate immunity, which is fast acting and temperature independent (Ellis, 2001) the predominant system of fish host defense. This innate immune response is essential for the survival of this whole class of animals. The defense includes many elements such as antimicrobial peptides (Cole *et al*., 1997) and polypeptides (Fernandes and Smith, 2002) non-classical complement activation, release of cytokines, inflammation and phagocytosis (Ellis, 2001; Magnadottir, 2006) Concisely, fish have evolved a number of innate immune responses to defend themselves against infection.

Source of AMPs

So far more than 750 different AMPs have been identified in various organisms ranging from insects to plants to animals including humans (Mendez *et al*., 1990; Liu and Hansen, 1990; Breukink and Kruijff, 1999; Schnapp *et al*., 1998). Besides these, bacteria themselves produce AMPs and about 50 of them have been isolated from various Gram-positive bacteria especially lactic acid-producing organisms (Luders *et al*., 2003) Most of these peptides are synthesized as a prepropeptide consisting of an N-terminal signal sequence (which aids in targeting of endoplasmic reticulum), a pro segment and a C-terminal cationic peptide that demonstrates antimicrobial activity after it is cleaved from the rest of the protein. These peptides have been grouped based on their primary structure, amino acid composition and their size.

Classification of AMPs

Nuclear Magnetic Resonance (NMR) has emerged as a useful technique for studying details of structures of most of the known antimicrobial peptides. Analysis of the three dimensional structure of these peptides has led to the better understanding of their function. Since a majority of these peptides are small in length, their three dimensional structures can be obtained using conventional two dimensional NMR methods. Based on the NMR structures of known peptides along with sequence analysis AMPs are broadly classified into five groups.

Helical AMPs

Much of the structural and biochemical work has been focused on Cecropins, which were the first to be identified and characterized (Steiner *et al*., 1981). All cecropins have helix-forming tendencies in certain organic co-solvents like Trifluoroethanol (Cammers-Goodwin *et al*., 1996) Initial studies with NMR showed that cecropin-A from *H. cecropia* exhibited a helical pattern in 15% Hexafluoroisopropyl alcohol (Holak *et al*., 1988). The results suggested a highly amphipathic helix with hydrophobic and cationic charged surfaces, a motif observed in many other AMPs. Magainins are another group of well characterized peptides composed of 23 residues isolated from the skin of the African clawed frog, *Xenopus laevis* (Matzusaki, 1999) NMR studies showed that like cecropins, magainins also form amphipathic -helical structures in 25% Trifluoroethanol (Marion *et al*., 1988).

Cysteine Rich AMPs

The human neutrophil peptides HNP-1, -2 and -3 were first of the cysteine-rich peptides isolated from the human granules (Ganz *et al*., 1985).These defensins are 30 amino acid peptides rich in cysteine residues and are present in a wide variety of organisms. Most of these defensin molecules harbour a consensus motif of six cysteine residues forming three in trimolecular disulfide bonds. The positions of the disulfide bridges are mostly between C1-C4, C2-C5 and C3-C6. X-ray crystallography studies with HNP-3, in combination with sedimentation equilibrium centrifugation, suggest that the peptide exists as a dimer . The NMR structure of defensin shows the presence of three-stranded antiparallel-sheets. Drosomycin, isolated from drosophila contain four disulfide bonds and are made up of three antiparallel strands with a helix in between the first two strands (Landon *et al*., 1997).

β-Sheet AMPs

A few of the known AMPs form a single hairpin structure and are approximately 20 residues long containing one or two disulfide linkages. Horseshoe crab peptides, tachyplesins and polyphemusin II, both share a hairpin motif stabilized by two disulfide bonds (Kawano *et al*., 1990; Tamamura *et al*., 1993). NMR studies along with 3D structures indicate that tachyplesin shows strong resemblance to protegrins, peptides isolated from porcine leukocytes. Both these molecules forms antiparallel -sheet connected to a turn and is composed of two disulfide bridges (Tamamura *et al*., 1993). NMR studies with thanatin isolated from the hemipteran insect *P. maculiventris* showed results similar to that of tachyplesin, including an antiparallel sheet maintained by a single disulfide bridge. Lactoferricin B, a 25 amino acid proteolytic derivative of lactoferrin in solution adopts a sheet structure stabilized by a single disulfide bond, as shown by NMR studies (Hwang *et al*., 1998). Schneider *et al*. (1998) have demonstrated that one can put together a novel combination of peptide synthesis modules and arrive at a novel structure.

AMPs Rich in Regular Amino Acids

Some AMPs are composed of high numbers of regular amino acids. The structural conformations of such peptides are different from the regular ά-helical or β-sheet peptides.

Histatin, a peptide isolated from human saliva is rich in histidine residues and is active against *C. albicans* (Xu *et al*., 2005) while cathelicidins are proline rich peptides and have irregular structures, indolicidins (Selsted *et al*., 1993) and tritripticin (Lawyer *et al*., 1996), are rich in tryptophan. Bactenecins Bac-5 and Bac-7, like cathelicidins, are proline-rich (Lawyer *et al*., 1996) while the peptide PR-39, is rich in arginine residues (Agerberth *et al*., 1991).

AMPs with Rare Modified Amino Acids

Few peptides are unusual as they are composed of rare modified amino acids. Best examples of such peptides are those produced by the bacteria themselves. Nisin, a lantibiotic, is one such peptide produced by *Lactococcus lactis* and is composed of rare amino acids like lanthionine, 3-methyllanthionine, dehydroalanine and dehydrobutyrine (Devos *et al*., 1993). The peptide is active against Gram-positive bacteria and shows no defined structural conformation in water, while it reveals several turn structures when bound to dodecylphosphocholine. Another peptide leucocin A, a 37-residue AMP isolated from *Leuconostoc gelidum*, is shown to form an amphiphilic conformation well suited for interacting with membranes (Fregeau *et al*., 1997). Such peptides undergo post-translational modification that result in conformations not seen in other classes of antimicrobial peptides. The gramicidins are composed of several DH-amino acids that allow them to form an unusual cyclic hairpin (Gibbs *et al*., 1998).

Mechanism of Action for Antimicrobial Peptides

Considering that AMPs are natural barriers to bacterial infections, pathogens ought to have developed a variety of strategies that render them resistant to antimicrobial host defenses. The only currently available structural model explaining the mechanism of action of AMPs Matzusaki (1999) explained the action of these peptides is from the outside and over the pathogen's membrane either by increasing their permeability or by destabilizing membranes by changing the net charge of the composed system. Since biological membranes are indeed dynamic fluids, the generation of resistance appears to be less likely to occur. Nonetheless, pathogens have evolved countermeasures not to resist, but at least to limit AMPs' effectiveness, such as chemical modifications and/or alternation of energy-dependent pumps at the membrane level. The same is true for intracellular bacterial pathogens, in which resistance-limitation is less effective against mostly cationic peptide-driven antimicrobial activity existing in the phagosomes of circulating monocytes, neutrophils and some mucosal epithelial cells. Additionally, the fact that the common features for most peptides are a net positive charge and an amphipathic nature, allows them to persist at water-lipid interfaces and then to disturb microbial membrane components (Ruissen *et al*., 2001).

Most of the peptides without disulfide bridges have random structures in water and it is only when they bind to a membrane or other hydrophobic environment, or self-aggregate, that these peptides form a structure. For example, cecropins and melittin fold into amphipathic alpha-helices in membranous environments. It is known that the dual cationic and hydrophobic nature of the peptides is important for the initial interaction between the peptide and bacterial membrane. Cationicity promotes interaction with bacterial outer and cytoplasmic membranes. Also, hydrophobicity is important and e.g., increasing the hydrophobic moment of magainin analogues causes increased binding of the peptide to the

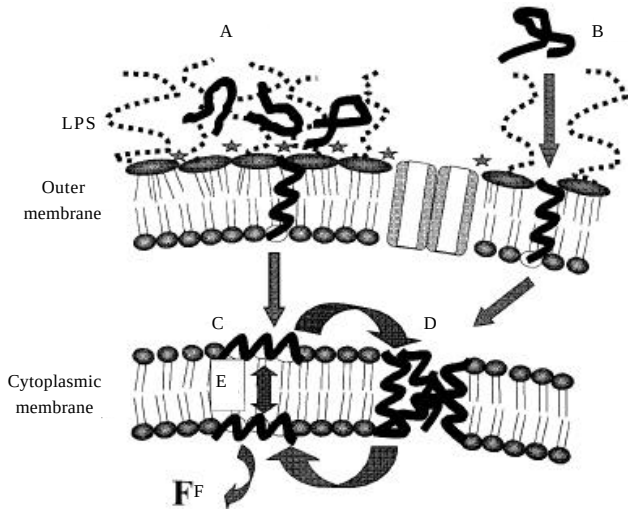

Fig. 1: The mode of interaction of antimicrobial peptide action in membrane system. Mechanism of interaction of cationic antimicrobial peptides with the cell envelope of gram-negative bacteria

membrane due to increased hydrophobic interactions between lipid acyl chains and the hydrophobic helix core (Wieprecht *et al*., 1997). An overview of the proposed interaction of peptides with the cell envelope membranes of gram-negative bacteria is given in Fig. 1.

The mechanism by which antimicrobial peptides act has become a complex issue. It is important to understand how the peptides act to fully exploit the use of peptides as antimicrobial agents. Small sequence changes can lead to major changes in activity. Not only is antimicrobial activity difficult to predict, but so are cytotoxic activities. Other peptides are selective for tumor over normal host cells. It is also very difficult to predict which peptides will be active *in vivo* based on *in vitro* MICs. However, many peptides do have reasonable activities in animal models without obvious toxicity (Hancock, 1997) and thus have been considered for potential use in the clinic route for passage of ions. To try to resolve such a dilemma arising from model membrane studies, have devised an assay based on measurement of the effects of peptides on the trans cytoplasmic membrane potential gradient. This assay showed that only certain peptides completely depolarize the cytoplasmic membrane of *Escherichia coli* at their MICs. However, they cause partial collapse of membrane potential at concentrations well below their MICs (an observation that contradicts the carpet model, which suggests that when peptides achieve a threshold concentration, the membrane is destroyed a phenomenon that is also not visible in electron micrographs). Still other peptides (e.g., indolicidin and bactenecin) do not permeabilize the cytoplasmic membrane to any great extent at their MICs and a separate mechanism of action is suggested. For different cationic peptides this has been proposed to be an action on the nucleic acids of bacteria or a triggering of autolysis. The bactericidal effects of these peptides tend to be extremely and therefore, it is difficult to monitor the stages of bacterial killing. Human lactoferrin peptides have a relatively slow action and for these peptides, it has been shown that membrane potential collapses, followed by membrane integrity, resulting in cell lysis. It has been also observed that the structures of human lactoferrin peptides alter with time once the peptides are bound to bacterial cell wall constituents and that the peptide does not form pores.

Antimicrobial Peptides Isolated from Fishes

The low infection rate of fish is remarkable and has inspired further studies of their innate defense system. Few antimicrobial peptides have been identified in fish. However, the number of microbial peptides being isolated and identified from the epidermal cells or secretion of the skin, gills and intestines of bony fish (teleosts) is constantly increasing. Some of these antimicrobial peptides have high sequence homology to known proteins with other function, suggesting a derivation from cleavage products of larger proteins, such as Histones (Park *et al*., 1998; Patrzykat *et al*., 2001; Birkemo *et al*. 2003; Fernandes *et al*., 2003, 2004) and ribosomal proteins (Fernandes and Smith, 2002). Peptides considered to be dedicated to the innate immunity have been isolated or cloned from fish and their expression has been analysed.

Fish have been largely ignored as a potential source of antimicrobial peptides. Of approximately 600 peptide antibiotics that have been isolated from various animals, relatively few have been identified from fish (Table 1).

A number of antimicrobial polypeptides and other defense components have been identified in many fish species. They includes natural antibiotics (Mendez *et al*., 1990) Apolipoproteins (Concha *et al*., 2003, 2004) a number of different isotypes of Muramidase (lysozyme) (Fernandes *et al*., 2004) permeability-increasing protein (Xu *et al*., 2005), Squalamine (Moore *et al*., 1993) and other unidentified antimicrobial factors (Ourth and Chung, 2004). However, sequences have been reported from only a minimal number of fish species (Douglas *et al*., 2003a, b), the largest vertebrate group containing over 23,000 species (Table 2).

Future Role of Antimicrobial Peptides as Therapeutic Agents

Microbial pathogens occupy and exploit a diverse variety of tissues and niches where they must confront antimicrobial peptide-mediated host defenses to survive. Thus, it is unrealistic to expect that no microbial pathogens are able to resist antimicrobial peptides. Rather, it is essential to understand whether a pathogen resists a given peptide and if so, through constitutive or inducible mechanisms. The main advantage of antimicrobial peptides

Table 1: Peptide antibiotics isolated from fish

Peptides	Fish	Approximate MW and No. of amino acids	Location	References
HFIAP	(Hagfish intestinal atlantic hagfish antimicrobial peptide)	3.5-4.6 kDa (30-37 AAs)	Intestine	Shinnar *et al*. (1996)
Pardaxins	Red sea moses sole	3.3 kDa (33AAs)	Skin (mucus glands)	Oren and Shai (1996)
Pleurocidins	Winter flounder	2.7 kDa (25 Aas)	Skin intestine	Cole *et al*. (1997), Douglas *et al*. (2001)
Piscidins	Hybrid striped bass,	2.5 kDa (22 Aas)	Skin, gill	Silphaduang and Noga (2001), Lauth *et al*. (2002)
Misgurin	Loach	2.5 kDa (21 Aas)	Whole fish	Park *et al*. (1997)
Hepcidins	White bass in other tissues	2.3 kDa (21 Aas)	Liver, low expression	Shike *et al*. (2002)
LCRP	Sea lamprey	2.2 kDa (19 Aas)	Skin	Conlon and Sower (1996)
Parasin-I	Asian catfish	2.0 kDa (19 Aas)	Skin	Park *et al*. (1998), Cho *et al*. (2002)
HSDF	Coho salmon	NR (26 Aas)	Mucus, blood	Patrzykat *et al*. (2001)

NR: Not reported; AA: Amino acids

Table 2: Antimicrobial polypeptides cloned from fish tissue

Peptides	Fish	Reference
Cathelicidins	Atlantic hagfish	Uzzell *et al*. (2003)
Cathelicidins	Rainbow trout	Chang *et al*. (2005)
Hepicidin	Red sea bream	Chen *et al*. (2005)

for innate defense is that they are small molecules and can be synthesized in a matter of hours, unlike components of adaptive immune response, which take days and can eliminate two or more intruders at the same time without requiring specific recognition for each foreign invader (Boman, 1995). As was mentioned earlier fish too possess antimicrobial peptides. In nature, where there are fewer stress factors present, their native antimicrobial peptides may be sufficient to protect fish against infections. However, in aquaculture facilities fish not only have to live in a plethora of microbes, but also encounter stress and physical injuries caused by other fish or the environment itself. These conditions set up the optimal circumstances for pathogens to prey on the susceptible host (Thune *et al*., 1993; Pickering, 1974).

Besides revealing the elevated level of host protection against pathogens, *in vivo* studies proved that insect borne diseases such as malaria can be prevented using insects carrying symbiotic bacteria transformed with an antimicrobial peptide gene and transgenic mice can be used to produce tracheal antimicrobial peptides, potential antibiotics for the treatment of cystic.

Fibrosis currently, antimicrobial peptides are in trials for their use in clinics for the treatment of skin infections associated with burns, diabetic wounds and eye infections (Boman, 1994, 1995). Evidently, the genes encoding these potent antimicrobial peptides represent good candidates for the genetic improvement of fish stocks to react to bacterial diseases.

CONCLUSIONS

Constitutive and inducible mechanisms of resistance to antimicrobial peptides are becoming clearer with the advent of genetically modified pathogens and the availability of reagent quantities of native or synthetic antimicrobial peptides. As with constitutive responses, it is not surprising that many of the mechanisms responsible for inducible resistance involve modifications of the pathogen envelope and/or extracellular facet of the cytoplasmic membrane directly offsetting mechanisms of peptide action.

In recent time has seen important progress in identification of new endogenous antimicrobial peptides and in the ascertaining of signals that can regulate their expression. The demonstration that a functional deficiency of endogenous antimicrobial peptides may contribute the persistent airway infections seen in patients with cystic fibrosis has increased attention and directed efforts to antimicrobial defenses of epithelial cells and mucous membranes. Expansion of work in this area should further clarify the effector mechanisms of innate immunity.

Recently marine peptides have opened a new perspective for pharmaceutical developments. The present review clearly shows antimicrobial peptides isolated from fishes would be a good source of antimicrobial agents and would replace the existing in adequate and cost effective antibiotics.

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