MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF ANTIMICROBIAL PEPTIDES IN MARINE FISHES

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Under the Faculty of Marine Sciences

By

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Molecular and Functional Characterization of Antimicrobial Peptides in Marine Fishes

Ph.D. Thesis under the Faculty of Marine Sciences

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Dedicated to...

All mothers and their sacrifices.....
All brothers and their concern and care.....
All fathers and the lessons they taught.....

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Certificate

This is to certify that the thesis entitled **"Molecular and Functional Characterization of Antimicrobial Peptides in Marine Fishes"** is an authentic record of research work carried out by Ms. Chaithanya E. R. under my supervision and guidance in the Department of the Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfilment of the requirements of the degree of Doctor of Philosophy under the Faculty of Marine Sciences of Cochin University of Science and Technology, and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution. All the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the Doctoral Committee have been incorporated in the thesis.

Kochi - 682 016
April 2016

Prof. (Dr.) Rosamma Philip
(Supervising Guide)

Declaration

I hereby declare that the thesis entitled **"Molecular and Functional Characterization of Antimicrobial Peptides in Marine Fishes"** is a genuine record of research work done by me under the supervision and guidance of Dr. Rosamma Philip, Professor, Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology and no part thereof has been presented for the award of any other degree, diploma or associateship in any University or Institution earlier.

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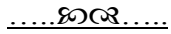


Chapter 1

GENERAL INTRODUCTION

Contents

1.1 Introduction

All living creatures, including the most primitive microbes to highly evolved and diversified mammals, possess a network of host defense mechanisms to thrive and establish in the austere external environment (Besse *et al.* 2015). Antimicrobial Peptides (AMPs) are such natural barriers of host innate immunity that are conserved throughout the course of evolution (Papagianni 2003; Reddy *et al.* 2004). AMPs are ribosomally synthesized, gene-encoded short peptides, which are either constitutively expressed or induced upon infections or stress as premiere artilleries of innate immunity (Reddy *et al.* 2004). They are widely distributed among living organisms ranging from prokaryotes to mammals including plants. More than 2400 AMPs have been isolated to date from various organisms spanning

around the phylogenetic spectra (http://aps.unmc.edu/AP/main.php). Despite the wide distribution and structural diversity, AMPs have some characters that are essential for biological activity in common. Generally, AMPs are small sized (10 kDa), amphipathic peptides with a net positive charge (Boman 2003). Although this is the classical concept about AMPs, AMPs having molecular weight greater than 10 kDa and net negative charge are also been reported from various organisms (Brogden *et al.* 2003; Wang *et al.* 2007; Haidong *et al.* 2013). As per AMP data base, approximately 90% of the reported AMPs are cationic in nature with an average net charge of +4 and hydrophobicity above 40% (Conlon 2015).

Generally AMPs are synthesized as an inactive prepropeptide or zymogen consisting of an N-terminal signal sequence, a pro segment and an N/C-terminal biologically active peptide (Bals 2000). The N-terminal signal peptide functions as a leader sequence to traverse endoplasmic reticulum whereas prodomain masks the catastrophic effects of the peptides to the host cell (Cai *et al.* 2012). Specific proteolytic cleavage of the N-terminal leader signal peptide and prodomain will result in the formation of the bioactive mature peptide (Hancock and Diamond 2000). Most of the AMPs exhibit wide spectrum antibacterial activity and that made them the principle effectors of primary defense as well as promising candidate in peptide antibiotic research (Zasloff 2002; Marshall 2003). Besides wide spectrum bactericidal activity, most of the AMPs exhibit antifungal, antiviral and anticancer properties also (Narayana and Chen 2015). When compared to immunoglobulins, AMPs are rapidly synthesized at low metabolic cost, and easily stored in large amounts in phagocytes, mast cells etc. They are readily available shortly after microbial infection and can

mobilize quickly to the point of infection and neutralize wide range of microorganisms (Papo and Shai 2003). This unique nature allied to the strategic location in phagocytes or epithelial surfaces and wide spectrum of activity against pathogenic microbes retain them as evolutionarily conserved and consistent throughout the course of evolution (Boman 2003; Reddy *et al.* 2004).

Commonly, AMPs are expressed in tissues that interface with external environment such as skin and mucosal epithelia of gill/lungs, intestine etc. to prevent the penetration and colonization of host tissue by infectious agents (Conlon *et al.* 2014). Furthermore, these peptides are stored in granules within phagocytes, mast cells and natural killer cells where they assist killing of engulfed microorganisms (Ganz and Lehrer 1998; Hirono *et al.* 2007) . Based on their ability to inhibit the growth of microbes, initially these peptides have been characterized as antimicrobial peptides; however multi-functional role of AMPs have also been recognized recently. They also function as effective modulators of the immune system, inducers of chemotaxis, activators of cytokine cascade and different immune cell types (Hancock *et al.* 2006; Conlon 2015).

Although AMPs have been recognized in the middle of 20th century, they have gained increasing attention in recent past only (Jenssen *et al.* 2006). Bacterial resistance to conventional antibiotics is a serious socio-economic concern and global public health problem. More than 70% of bacteria are resistant to at least to one antibiotic prescribed today and some of them are multiple drug resistant also (Takahashi *et al.* 2010). This led the scientific community to search for novel therapeutic agents, which can

overcome bacterial resistance. Here comes the significance of AMPs, which are natural antibiotics produced by almost all organisms and can neutralize wide range of microbes without developing any resistance. AMPs are promising candidates in the field of pharmacology and medicine as novel therapeutic agents that could overcome bacterial resistance (Boman 2003; Giuliani *et al.* 2007; Maróti *et al.* 2011). The same is the reason for increased popularity and intensive research in the field of peptide antibiotics.

AMPs exhibit some general features like small size, amphipathic nature and net positive charge; they are highly diverse in structural organization, amino acid composition and mechanism of action. There is no well-defined standard classification for AMPs since it is a relatively emerging field of science. AMPs are classified by various authors in diverse ways. Classification of AMPs based on their secondary or tertiary structure and mode of action on target cell membranes are the two most accepted classifications of AMPs.

1.2 Classification of AMPs

1.2.1 Structural classification of AMPs

Most of the AMPs are unstructured in aqueous solutions and they can assume specific structures only in membrane mimetic environments or in close proximity with microbes (Nissen-Meyer and Nes 1997). Depending upon the amino acid composition, AMPs can adopt various secondary structures that are least energetic and stable. Based on the secondary structure, AMPs could be broadly classified into three large groups, namely α-helical peptides, cysteine-rich β-sheet peptides, and flexible

peptides rich in certain amino acids such as tryptophan, proline, arginine, histidine, and glycine (Andreu and Rivas 1998; Douglas *et al.* 2003a; Papagianni 2003; Reddy *et al.* 2004; Bjorstad 2009; Takahashi *et al.* 2010).

a) α-helical peptides

Peptides with α-helical conformation are among the most characterized peptides and accounts for 2/3rd of the total isolated AMPs. Generally they have 12-40 amino acid residues in length and usually rich in helix-stabilizing residues like alanine, leucine, lysine with no cysteine. Many of these AMPs are not strictly α-helical alone, but a flexible unstructured segment at the N- and/or C-terminus and often an internal kink are also found as a common feature of most α-helical peptides (Takahashi *et al.* 2010). For example, human cathelicidin LL-37 is an α-helical peptide composed of two helical regions with a glycine-induced minor kink near the center and a flexible segment at the C-terminus (Takahashi *et al.* 2010). Cecropins (Hultmark *et al*. 1980; Steiner *et al*. 1981) and melittin (Terwilliger and Eisenberg 1982) from insects, magainins from frogs (Gesell *et al.* 1997), cathelicidins of mammals, fishes and fowl (Maier *et al*. 2008; Zanetti *et al*. 1995; Xiao *et al*. 2006), piscidins (Angelis *et al.* 2011; Olivieri *et al.* 2015; Umasuthan *et al.* 2015) and hipposin (Birkemo *et al.* 2003) from fishes are peptides that can assume α-helical conformation in membrane mimetic environments.

b) Cysteine-containing β-sheet peptides

These are peptides rich in cysteine residues and are frequently comprised of several antiparallel β-sheets stabilized by 1-6 disulphide bonds between the cysteine residues (Yount *et al.* 2006; Bjorstad 2009).

Mammalian/fish/insect and plant defensins (García *et al.* 2001; Lopez *et al.* 2003; Hanks *et al.* 2005; Casadei *et al.* 2009; Nam *et al.* 2010), vertebrate hepcidins (Shi and Camus 2006; Segat *et al.* 2008; Lee *et al.* 2012a), porcine protegrins (Steinberg *et al.* 1997; Ishitsuka *et al.* 2006), horseshoe crab tachyplesins (Kushibiki *et al.* 2014) and polyphemusin II (Tamamura *et al*. 1994) are some of the examples of peptides that could form β-sheet/β-hairpin peptides. Majority of β-sheet peptides belong to the defensin family. Defensins are the most diverse and widely distributed AMPs of both vertebrates and invertebrates to a larger extent (Ganz and Lehrer 1998; Dimarcq *et al.* 1999). In all cysteine-rich peptides, there is a motif called γ-core formed of two antiparallel β-sheets with basic residues polarized on its axis as the basic unifying structural archetype and is essential for biological activity also (Yount *et al.* 2006).

c) Flexible AMPs rich in certain amino acids

Certain AMPs are composed of high numbers of regular amino acids and the structural conformations of such peptides are different from the regular α-helical or β-sheet peptides. Histatin, a peptide isolated from human saliva is rich in histidine residue (Xu *et al*. 1991), armadillidin rich in glycine (Braquart-Varnier *et al.* 2005), indolicidins (Selsted *et al*. 1992) and tritripticin (Lawyer *et al*. 1996) are rich in tryptophan, while cathelicidins (Anderson and Yu 2003) and penaeidins (Li and Song 2010) are rich in proline. Some other AMPs are rich in modified or unusual amino acids. 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 (de Vos *et al.*

1993). Because of the unusual amino acid composition, these peptides adopt highly variable structures. For example, 13-amino acid indolicidin adopts a largely extended wedge shaped conformation on membrane mimetic environment (Rozek *et al.* 2000a; Yount *et al.* 2006). Similarly gramicidin exhibits an unusual cyclic β-hairpin like structure due to the presence of DH-amino acids (Gibbs *et al.* 1998).

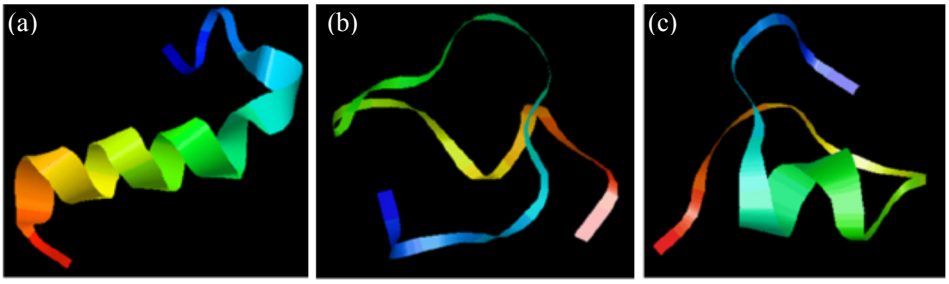


Fig. 1.1: a) α-Helical antimicrobial peptide, b) Cysteine containing β-sheet peptide and c) Flexible AMP rich in certain amino acid.

1.2.2 Functional classification of AMPs

Though AMPs have been isolated from wide range of organisms covering the phylogenetic spectrum (Papagianni 2003), the exact physiological function and mode of action of these host defense peptides is still a matter of controversy even decades after the discovery of the first antimicrobial peptide, cecropins (Steiner *et al.* 1981). A number of models have been put forward to explain the mechanism of action of AMPs. But, based on the mode of action on lipid membranes, AMPs could be classified into two mechanistic classes: 1) membrane disruptive (barrel stave, toroidal, carpet and micellar aggregate) and 2) non-membrane disruptive (intracellular targets) models (Nissen-Meyer and Nes 1997; Yount *et al.* 2006).

1.2.2.1 Membrane disruptive mode of action of AMPs

Though there is still ambiguity in the mechanism of action of AMPs, most workers have unique opinion about the membrane lytic potentials of these peptides. Most of these peptides selectively disrupt the microbial cell membranes and ultimately lead to the death of the organism (Sato and Feix 2006). Microbial outer membrane is rich in negatively charged moieties like lipopolysaccharide (LPS), teichoic and teichuronic acids and are enriched in acidic phospholipids such as phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL), conferring an overall net negative charge (Yeaman and Yount 2003). Similarly, phosphomannans or related constituents often create electronegative aspects of fungal surfaces (Yount *et al.* 2006). Cationic AMPs primarily attach electrostatically to the negatively charged microbial cell membranes and disintegrate membrane equilibrium or create trans-membrane pores through which cellular content oozes out and eventually leads to the death of the organism (Giuliani *et al.* 2007). Recent review portrayed AMPs as specific natural antibiotics with broad spectrum antimicrobial activity. Except few, most of these peptides are non-cytotoxic to mammalian erythrocytes. The specificity of AMPs towards microbes is on account of their anionic membrane constituents. When compared to microbial cell surface potential, mammalian cells are neutral in charge due to the presence of zwitter ionic moieties and even if present, anionic lipids are arranged along the cytoplasmic side of the membrane. This will result in masking its charge on surface. Furthermore, presence of cholesterol neutralizes the membrane charge and limits its action on the mammalian membrane. This is the reason for the specificity of AMPs (Reddy *et al.* 2004; Hoskin and Ramamoorthy 2008). The initial attraction is dependent on

various structural determinants like amphipathicity, helical property, charge of the peptide, structure and amino acid composition (Nissen-Meyer and Nes 1997; Khandelia *et al.* 2008; Guaní-Guerra *et al.* 2010). A moderate positive charge, hydrophobicity and capacity to adapt helical conformation on membrane mimetic environments are essential requirements of an AMP to traverse across the membrane. As the hydrophobicity and charge increased above a certain level, they force a strong partition of the peptide into the hydrophobic core of the lipid bilayers, regardless of the phospholipid head group, thereby resulting in an increased AMP adsorption and antimicrobial activity; but this could be achieved at the cost of decreased selectivity and cytotoxicity only (Strömstedt *et al.* 2010; Manzini *et al.* 2014; Schmidtchen *et al.* 2014). Two distinct channel architectures have been described to explain the pore formation. They are barrel-stave model that involves the formation of trans-membrane ion channels, and a toroidal-pore model (Hancock and Diamond 2000; Reddy *et al.* 2004; Jenssen *et al.* 2006). Carpet model or micellar aggregate models explains non-pore forming rupture of the cell membrane by AMPs (Sato and Feix 2006; Takahashi *et al.* 2010).

a) Ion channel formation - Barrel Stave model

Barrel stave model explains how positively charged AMPs get attracted towards anionic microbial outer membranes and forms trans-membrane pores lined by AMPs similar to staves in a barrel. According to this model, amphipathic α-helical peptides come to contact with microbial membranes as a result of electrostatic attractions and align themselves in such a way that their hydrophobic sides are facing the phospholipid acyl chains and their hydrophilic surfaces lining a water-filled channel like ‘staves’ in a ‘barrel’-

shaped cluster which is perpendicular to the plane of the membrane (Jenssen *et al.* 2006). Only AMPs with sufficient length could form barrel-staves. Unless and otherwise the peptides are sufficiently long enough to traverse the hydrophobic core of the bilayer, they cannot form ion channels (Reddy *et al.* 2004; Sato and Feix 2006).

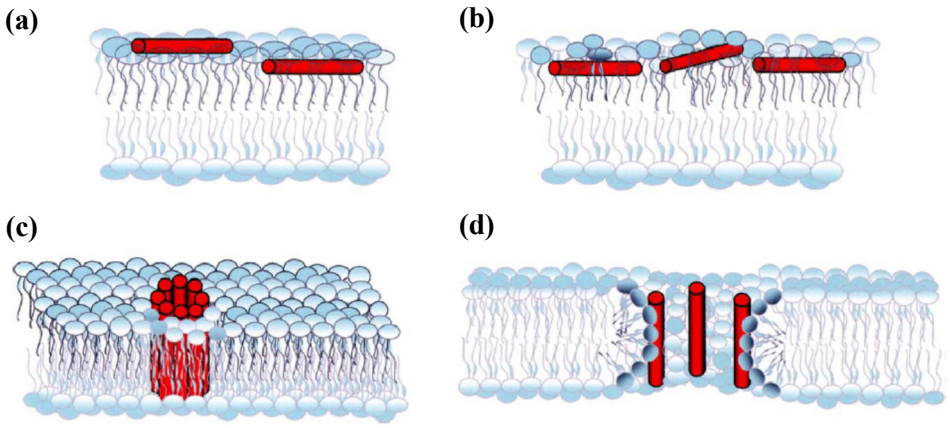


Fig. 1.2: Pictorial representation of the mechanism of trans-membrane pore formation. (a) The peptide monomers binds electrostatically to the membranes, initially parallel to the membrane in an α-helical confirmation, followed by the localization of more peptide molecules on the cell membrane (b) Peptides continue to accumulate at or near the bilayer surface, disrupting lipid packing and causing membrane thinning. Once a critical peptide/lipid ratio is reached, peptides either (c) insert into the membrane as a barrel-stave type pore, or (d) induce the localized formation of toroidal pores (Adopted from Sato and Feix 2006)

b) Toroidal-pore model

This model was first proposed based on studies with magainin and the pores formed by the peptides were of approximately 30 to 50 Å in diameter (Ludtke *et al.* 1996). AMP induced expansion of the lipid head

group region results in a positive curvature of the membrane, which in turn facilitates formation toroidal pores. The toroidal-pore model differs from barrel-stave model in the nature of pores only. The pore formed according to this model is lined by both peptides and membrane lipids, whereas pores formed by barrel-stave model is lined by peptide monomers alone (Sato and Feix 2006). Once a transient pore is formed across the membrane osmotic gradient, regardless of the nature of the pore, cell swelling will occur and thus facilitate further thinning of the lipid bilayer. Such changes in the physical properties of the membrane may also enhance peptide binding and creating a disruptive cycle that eventually leads to cell lysis or death. Gram-positive bacteria are more resistant to AMPs which interact with bacterial membranes by pore formation-mediated killing because of the ability to withstand the turgor pressures 3 to 25 times higher than those tolerated by Gram-negative bacteria owing to the presence of an additional cell wall (Koch *et al*. 1987).

c) Carpet model

This mechanism was first described by Steiner and co-workers in 1988 to describe the mechanism of action of cecropin and its derivatives. The model explains a non-pore forming membrane disruptive mechanism in which peptides accumulate at the bilayer surface like a carpet. Above a particular threshold concentration of monomers, the membrane is permeated and disintegrated without the formation of discrete pores or channels in a detergent-like manner. Although the carpet and channel/pore-forming models have a number of differences, they also share some characters in common. Both types of mechanisms begin with electrostatic attraction of

AMP to the membrane surface and subsequent accumulation of the peptide to a critical concentration. In barrel-stave and toroidal pore forming models, AMP monomers penetrate into the lipid core while in carpet model, individual peptides do not penetrate into the lipid core; instead destabilize the membrane equilibrium and leads to the micellization of the membrane. In all cases, the cell lysis is due to osmotic imbalance initiated collapse of the cell. Toroidal pores are regarded as an intermediate state prior to micellization/formation of "holes" or toroidal pores may occur as an early step in membrane disintegration (Gazit *et al*. 1995; Shai 2002). Membrane permeabilization alone is sufficient to induce membrane lysis, without progression to complete membrane destruction (Sato and Feix 2006).

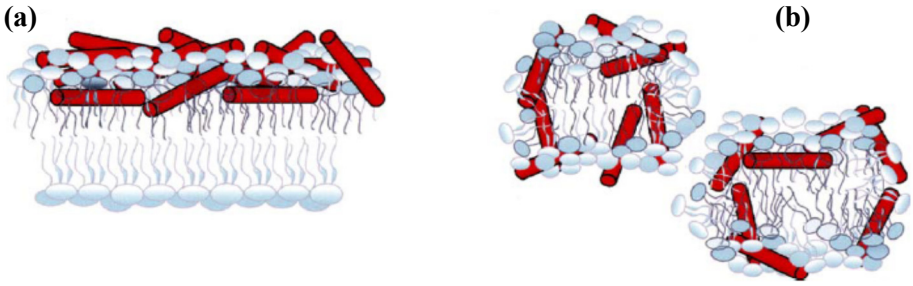


Fig. 1.3: Pictorial representation of carpet-like mechanism of action of AMPs. (a) Peptides get attached to the bilayer like a carpet (b) Disintegration and micellization of membrane by cationic AMPs. (Adopted from Sato and Feix 2006)

1.2.2.2 Non-membrane disruptive mode of action of AMPs/ Internal targets of AMPs

A number of intracellular targets that may hinder essential biosynthetic and metabolic functions of the cell have been identified for AMPs in recent past beyond membrane permeabilization and subsequent death (Yount *et al.* 2006; Lan *et al.* 2010). They could penetrate and

accumulate within the cytoplasm of the infectious agent, where they target variety of cellular processes. This includes impairment of cell wall assembly, cellular organelle, nucleic acid synthesis, mutation of essential enzymes and quorum sensing proteins. Lantibiotic nisin and mersacidin interferes with trans-glycosylation of lipid II, an intermediate step in peptidoglycan synthesis and thereby inhibits bacterial cell wall synthesis (Brotz *et al.* 1997). Insect antibacterial peptides pyrrhocoricin, drosocin and apidaecin bind to the bacterial heat shock protein DnaK and inhibit the ATPase activity of the protein, which will in turn prevent chaperon-assisted folding of the proteins and results in the accumulation of misfolded proteins and death (Otvos *et al*. 2000; Kragol *et al*. 2001). Binding and inactivation of poly anionic nucleotides by cationic AMPs are one important mechanism by which AMPs prevent microbial growth and multiplication. Pleurocidin (Patrzykat *et al.* 2002), dermaseptin and buforin II were found interacting with DNA/RNA resulting in the subsequent killing of pathogens (Xie *et al.* 2011). Some AMPs like microcin B17 inflicts mutation in DNA gyrase and impair nucleic acid synthesis (del Castillo *et al*. 2001). Certain other peptides like microcin B17 specifically target DNA gyrase and interfere with normal DNA function. Another antimicrobial indolicidin, a tryptophan-rich peptide isolated from bovine granulocytes, functions through binding and/or inactivation of DNA. Indolicidin will permeabilize native and artificial membranes without inducing lysis or vesicle leakage and binds to DNA and inactivate it (Subbalakshmi and Sitaram 1998). In fungi, cellular organelles such as mitochondria are also targeted in a similar way as bacteria are treated by the peptides, indicating the significance of

endosymbiont hypothesis. AMPs are multifunctional peptides with variable targets depending upon the concentration of the peptide, nature of target membrane and the physical properties of the interacting membrane (Jenssen *et al.* 2006).

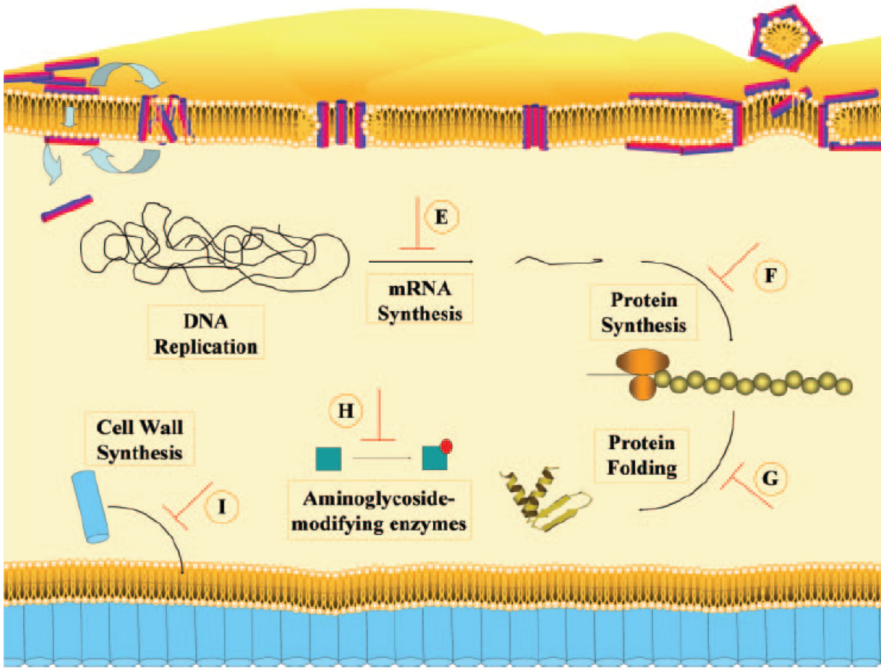


Fig. 1.4: Intracellular targets of Antimicrobial Peptides (Adapted from Jenssen *et al.* 2006)

1.3 Biological functions of AMPs

1.3.1 AMPs as antibacterial agents

Most of the known AMPs are broad spectrum antibacterial agents even at micromolar concentrations. Probably, the most studied biological function of AMPs could be its bactericidal potential. Most of the AMPs are cationic with an average value of +4 (Strömstedt *et al.* 2010). This will

help the peptide to get electrostatically attached to the negatively charged bacterial membranes (Nissen-Meyer and Nes 1997; Papagianni 2003). Non-specific physical disruption of bacterial outer membrane and/or cytoplasmic membrane either through pore formation or depolarization of membranes with effective replacement of divalent cations are found to be the primary mode of amicrobial action (Reddy *et al.* 2004; Takahashi *et al.* 2010). Human saliva, urine, sweat, several vertebrate tissues, frog and fish skin mucus, arthropod haemolymph, mollusc mucus etc. are rich sources of antibacterial peptides (Bachère *et al.* 1995; Anderson and Beaven 2001; Krause *et al.* 2003; Saito *et al.* 2005; Smet and Contreras 2005; Urban *et al.* 2007). Besides, even bacteria and extremophiles produce antibacterial peptides for the survival and effective utilization of available niches (Pokusaeva *et al.* 2009; Besse *et al.* 2015).

1.3.2 AMPs as antifungal agents

More than 1.5 million fungal species have been identified so far. Among these, a few hundred including yeast pathogens, opportunistic moulds and dermatophytes have been concomitant with human diseases (Ajesh and Sreejith 2009). Similar to bacterial cell wall/membrane, fungal cell wall is also rich in chitin and other negatively charged moieties (Narayana and Chen 2015). Antifungal peptides are capable of efficient binding to chitin and subsequent cell rupture (Pushpanathan *et al*. 2012). Many of the plant and animal- defensins (Rogozhin *et al.* 2011), insect-cecropin, drosomycin (de Lucca *et al.* 1997; Michaut *et al.* 1996), wasp venom peptide- decoralin (Konno *et al.* 2007) and anoplin (Konno *et al.* 2001), frog/toad skin peptides- magainin (Bechinger and Salnikov 2012),

dermaseptin (Lazzari and Bloch 2007), brevinin (Pal *et al.* 2006), fish derived peptides - epinecidin (Pan *et al.* 2010), pleurocidin (Jung *et al.* 2007), hepcidin (Hunter *et al.* 2002), human secretory peptides- lactoferrin and histatin are potential antifungal peptides (Ajesh and Sreejith 2009). Many of these AMPs exhibit promising activity against potent yeast and fungal pathogens like *Candida albicans, C. tropicalis, C. krusae, C. parapsilopsis, Fusarium graminearum, F. oxysporum, Cryptococcus neoformans, Saccharomyces cerevisiae, Pichia membranifaciens, Aspergillus* sp. etc. (Ajesh and Sreejith 2009).

1.3.3 Antiviral AMPs

When compared to anti-bacterial and anti-fungal peptides, antiviral peptides are less numerous. But some of the peptides exhibited promising activity against lethal viral aquaculture pathogens as well as deadly human pathogens like human immunodeficiency virus (HIV) (Hancock and Diamond 2000; Hancock *et al.* 2006). Defensins, a widely distributed AMP in nature, are able to neutralize herpes simplex virus (HSV), vesicular stomatitis virus, influenza virus, viral hemorrhagic septicemia virus (VHSV) and newly emerged fish viruses like Singapore grouper irido virus (SGIV) and viral nerval necrosis virus (VNNV) (Daher *et al*. 1986; Ganz and Lehrer 1998; Falco *et al.* 2008; Guo *et al.* 2012). Frog skin peptides, magainin, brevinin and dermaseptin are effective inhibitors of HSV type 1 and 2. Dermaseptin has exhibited promising activity against acyclovir-resistant HSVs with an EC50 $\leq$ 6 μM (Lazzari and Bloch 2007; Conlon *et al.* 2014). Tachyplesins and polyphemusins, two peptides identified from horse-shoe crab are active against vesicular stomatitis virus,

influenza-A virus and HIV (Tamamura *et al*. 1993). Insect AMPs like melittins and cecropins also displayed anti-HIV activity by suppression of the long-terminal repeat gene (Wachinger *et al*. 1998). Reports of fish derived peptides with antiviral property are very less. However, epinecidin and tilapia hepcidin 1-5 were found active against nervous necrosis virus both *in vitro* and *in vivo* (Wang *et al.* 2010). Nevertheless some AMPs exhibited encouraging results with both aquaculture as well as human pathogenic viruses; the exact mechanism of antiviral action is yet to be discovered.

1.3.4 Anticancer AMPs

Cancer has emerged as a major public health concern in developing countries. According to WHO, death rate due to cancer will increase up to 11 million/year by 2030. Nevertheless, promising progress has been achieved in respect of cancer therapies (Riedl *et al.* 2011). Potential toxicity of conventional chemotherapeutics against normal cells (due to less specificity) and multiple drug resistance of cancer cells have led scientific community to search for novel specific non-resistant chemotherapeutic agents with less toxicity (Chu *et al.* 2015). Many of the antimicrobial peptides which are toxic to bacteria and nontoxic to normal mammalian cells, exhibit a broad spectrum of cytotoxic activity against cancer cells (Lee *et al.* 2008; Feliu *et al.* 2010; Chi *et al.* 2015). Fundamental differences exist between the cell membranes of malignant cells and normal cells. Electrostatic attraction between the negatively charged components of cancer cells and the positively charged AMPs is believed to play a major role in the strong binding and selective disruption of cancer

cells. Furthermore, higher cell surface area of the cancer cells due to the presence of increased number of microvilli also enhance the lytic activity of anticancer peptides (ACPs) by facilitating membrane destabilization (Domagala and Koss 1980). Many ACPs/AMPs from various sources have exhibited promising anticancer activity against various cancer cell lines including fish cell lines. The skin secretions of amphibians are a rich source of AMPs, several of which (aurein, citropin, gaegurins, temporin, magainins and analog peptides of magainins) have been reported to be selectively cytotoxic for human cancer cells (Cruciani *et al*. 1991; Park *et al.* 1994; Rozek *et al*. 2000b; Doyle *et al*. 2003; Wang *et al.* 2012a). A number of synthetic magainin analogues, insect cecropins (Kao *et al.* 2012), melittin (Barrajón-Catalán *et al.* 2010), Polybia MP1 (Wang *et al.* 2008), shrimp anti-lipopolysaccharide factor (Lin *et al.* 2010), molluscan peptides (Chi *et al.* 2015), fish derived peptides (Tilapia hepcidin TH1-5, epinecidin and pardaxin) (Chen *et al.* 2009a; Hsieh *et al.* 2010; Chang *et al.* 2011; Hsu *et al.* 2011b), mammalian lactoferrin (Tomita *et al*. 2002) and human salivary peptides (da Costa *et al.* 2015) exhibit *in vitro* cytotoxic activity against many different types of mouse and human cancer cell lines, including leukemia cells, fibrosarcoma cells, various carcinomas, and neuroblastoma cells at concentrations that do not substantially affect the viability of normal fibroblasts, lymphocytes, epithelial and endothelial cells, or erythrocytes (Rajanbabu and Chen 2011a; Thundimadathil 2012; Masso-Silva and Diamond 2014). In recent years, a number of attempts have been made to improve upon naturally occurring ACPs by creating hybrid ACPs that incorporate the best qualities of individual ACPs (Wu *et al.* 2009; Fox *et al.* 2012; Kao *et al.* 2012). Emerging results uphold

potential usage of AMPs in various aspects of cancer treatment including targeted chemotherapy, drug delivery and even as vaccines against cancer (Thundimadathil 2012; Huang *et al.* 2013).

1.3.5 Other aspects of AMPs

Apart from antimicrobial activities, few peptides have been explored for their contraceptive potential. The frog skin peptide, magainin caused 100% sperm immobilization at a concentration range of 50 to 800 μg in mammals including humans (Reddy *et al.* 2004). Another magainin analogue, magainin-A inhibited conception when applied intra-vaginally at the dose level of 200 μg in rat and 1 mg in rabbit and monkey (Aranha *et al.* 2004). Besides, the peptide is embryo toxic and prevents pre-implantation of mouse embryos in *in vivo* studies (Sawicki and Mystkowska 1999). Nisin, a bacteriocin at a concentration of 300–400 μg/ml was found to be sufficient to inhibit human sperm motility within 20 s *in vitro* and *in vivo* (Reddy *et al.* 2004). Vaginal administration of 1 mg of nisin stopped sperm motility and pregnancy in rabbits completely (Reddy *et al.* 2004). Contraceptive potential of AMPs is a relatively unexplored field which requires better attention and further research.

1.4 AMPs at Clinical trials

Despite of all promising attributes as novel antimicrobial agents, only few have reached clinical trials (Narayana and Chen 2015). Ambiguity in apparent toxicity of the peptides *in vivo*, stability, specificity and improper drug delivery are the major challenges in the usage of AMPs *in vivo* as therapeutics (Silva *et al.* 2013). Effective drug delivery systems

such as liposomes and introduction of unusual amino acids (mainly D-form amino acids) or modification of the terminal regions (acetylation or amidation) would reduce the toxicity and improve the stability of the peptide (McPhee *et al*. 2005; Samad *et al*. 2007; John *et al*. 2008). Most pharmaceutical efforts have been devoted to the development of topically applied agents largely because of the above reason and relative safety of topical therapy (Zasloff 2001). Pexiganan, a 22-aminoacid analogue of magainin 2, was the first antimicrobial peptide to undergo commercial development in the trade name LocilexTM (Gaenera, USA), as an antibiotic cream for the topical treatment of diabetic foot ulcers (Giuliani *et al.* 2007). Two indolicidin-based peptide variants, MX-594AN (Omiganan pentahydrochloride 1% gel) and MX-226 developed by Migenix (Canada) have completed phase IIb and IIIb clinical trials for acne catheter related infections (Giuliani *et al.* 2007; Narayana and Chen 2015). Neuprex (Xoma, USA) is the trade name of a modified recombinant bacterial permeability increasing protein (BPI) currently under preclinical trials against trauma and surgery-related infections and diseases (Domingues *et al*. 2012). P113D is a licensed formulation by Par Advance Technologies, Inc. for the treatment of oral candidiasis in HIV patients. It is a 12-amino-acid cationic peptide based on human salivary histatin with D aminoacid modification (Cummins *et al*. 2007). A 11 aa human lactoferricin-derived peptide hLF-1-11 is under preclinical trials for endotoxin and fungal related infections (Costa *et al*. 2014). Plectasin, the first defensin ever found in fungi, is a promising AMP with biological activity comparable to vancomycin and penicillin against systemic pneumococcal infections and is undergoing preclinical trials now (Water *et al*. 2015). Eventhough the therapeutic potential of AMPs are

recognized well, pharmacology and pharmacokinetics of AMPs are still at infancy.

1.5 AMPs from fishes and their significance

Fishes are the largest as well as earliest class of vertebrata. They are adapted to live in highly diversified and extreme aquatic milieus. Recent literature recognizes fishes as rich source of antimicrobial peptides as they heavily depend upon the primeval innate defense (Magnadottir 2006). Mucosal epithelial layer acts as a primary physical barrier to many aquatic organisms, especially for fishes as a protection from the microbe-laden external environment. Skin secretions of fishes contain many antimicrobial substances including AMPs. In the beginning many of the peptides were identified and purified from skin secretions and body fluids. Later, advancement in bioinformatic tools paved the way for the identification of numerous novel peptides by relatively effortless means (Masso-Silva and Diamond 2014). The first identified fish AMP was pardaxin (Primor and Tu 1980). Pleurocidin (Cole *et al.* 1997), oncorhyncin (Smith *et al.* 2000a), hipposin (Birkemo *et al.* 2003), moronecidin (Lauth *et al.* 2001), and parasin (Park *et al.* 1998b) are AMPs identified and characterized from the skin secretions of fish. Besides skin secretions, AMPs have been identified from almost all tissues of fishes including nervous and connective tissues (Silphaduang *et al.* 2006). These AMPs are expressed in fish either constitutively or inductively upon microbial infection (Bulet *et al*. 2004). Many of them even play key role in cell mediated immunity and phagosome-assisted killing of antigens also (Mulero *et al.* 2008; Dezfuli *et al.* 2010).

Similar to previously reported AMPs, fish AMPs are also predominantly cationic (average cationicity +4), amphipathic, small sized peptides of less than 10 kDa (Conlon 2015). About 60% of fish AMPs identified so far are comprised of cysteine containing β-sheet peptides and the rest is contributed by α-helical AMPs (Masso-Silva and Diamond 2014). Many of the AMPs present in fishes are found expressed in mammals also. The AMPs cathelicidins, defensins and hepcidins are uniformly distributed among lower as well as higher vertebrates. Remarkable structural and functional similarity of AMP genes between fishes and higher vertebrates suggests the possibility of common ancestor as well as expansion and diversification of gene products probably by gene and genome duplication events (Zou *et al.* 2007). Sorting of novel bioactive peptides through the depths of ocean and extreme environments still remains unattractive and unexplored (Patrzykat and Douglas 2003). Hitherto, more than 100 AMPs have been identified from various fishes.

1.5.1 AMPs from Agnatha

The jawless fishes are the first vertebrate stock on earth and evolutionary forerunners of modern day fishes (Conlon 2015). However, they were abundant during Silurian and Devonian ages; hagfishes and lampreys are the only surviving members of present day cyclostomes. Adaptive immunity is less/feebly developed in cyclostomes and hence they mainly depend on innate immune parameters (Buchmann 2014). Under threat, hagfishes and lampreys produce large amount of slime. Recent research reveals that this slime contains various lysozyme and host defense

peptides (Subramanian *et al.* 2008). Myxinidin is a 12 aa AMP identified from the skin mucus extract of hagfish *Myxine glutinosa* (Subramanian *et al*. 2009). It is a non haemolytic amphipathic α-helical peptide with broad spectrum antibacterial and antifungal activity (Cantisani *et al.* 2014). Three AMPs (HFIAP-1, 2, and 3) similar to mammalian cathelicidins were identified from hagfish intestinal tissues (Uzzell *et al.* 2003). Another AMP structurally similar to mammalian corticostatins has also been reported from cyclostomes (Conlon and Sower 1996). Besides, antimicrobial, antifungal as well as anti-parasitic peptides, shark aminosterol squalamine were also identified from white blood cells of lamprey challenged with bacterial suspension (Yun and Li 2007). Presence of squalamine and peptides related to mammalian cathelicidins and corticostains in primitive vertebrates like cyclostomes indicate evolutionary significance and preservation of AMPs through the course of evolution (Conlon 2015).

1.5.2 AMPs from Elasmobranchs and other primeval orders

Elasmobranchs are best-known for the aminosterol antibiotic compound squalamine (Moore *et al*. 2003). They are relatively less exploited source in terms of AMPs. A 28 aa strongly cationic peptide termed kenojeinin with activity against both Gram-positive and Gram-negative bacteria and fungus was identified from the fermented skate (*Raja kenojei*) skin (Cho *et al.* 2005). Recently two histone-derived AMPs himanturin from round whip ray (*Himantura pastinacoides*) and harriottin 1, 2 & 3 from Sickle fin chimaera (*Neoharriota pinnata*) have also been characterized (Sathyan *et al.* 2012a, 2013).

AMPs are yet to be characterized from ancient fish orders like Holostei, Chondrostei and Dipnoi. However, a N-acetylated histone H2A fragment with promising antimicrobial activity have been recorded from leucocytes of the Russian sturgeon *Acipenser gueldenstaedtii* (Conlon 2015). Ancient fishes including Elasmobranchs are potential sources of AMPs. But further investigation in this regard has to be carried out to better understand immune system and related parameters of these fishes.

1.5.3 AMPs from Teleosts

Teleostei is the most successful branch of fishes with maximum number of living representatives. Nevertheless, fishes sill remain as underexploited source of AMPs. They have the potential for development into therapeutically valuable drugs (Conlon 2015). Majority of the fish-derived AMPs reported to-date are characterized from either fresh water or cultured fishes with few exceptions only. This includes fresh water carps (Li *et al.* 2013; Yang *et al.* 2014), Loaches (Nam *et al.* 2011), Catfishes (Bao *et al.* 2006; Ting *et al.* 2014), Snake head (Gong *et al.* 2014), Zebrafish (Zou *et al.* 2007), Trouts (Smith *et al.* 2000; Fernandes *et al.* 2004; Chang *et al.* 2005), Salmon (Richards *et al.* 2001; Douglas *et al.* 2003a), Seabass (Ren *et al.* 2006; Meloni *et al.* 2015), Sea-breams (Martin-Antonio *et al.* 2009; Umasuthan *et al.* 2015), Cods (Fernandes *et al.* 2010; Ruangsri *et al.* 2012), Groupers (Yin *et al.* 2006; Mao *et al.* 2013), Croaker (Li *et al.* 2014), Flatfishes (Cole *et al.* 1997; Arma *et al.* 2005) and Antarctic ice fishes (Xu *et al.* 2008; Buonocore *et al.* 2012). Defensins, cathelicidins, piscidins, hepcidins and histone-derived peptides are the major groups of AMPs characterized from fishes (Masso-Silva and

Diamond 2014) among which piscidins and hepcidins are the most abundant and diversified peptides due to positive Darwinian selection (Padhi and Verghese 2007; Fernandes *et al.* 2010) and adaptive evolution.

Most of the fish-derived AMPs exhibit wide spectrum of antibacterial activity against *Streptococcus, Staphylococcus, Bacillus, Enterobacter, Salmonella Pseudomonas, Aeromonas, Klebsiella, Yersinia* and *Vibrio* species (Masso-Silva and Diamond 2014). Besides bactericidal property, many of them especially piscidins and hepcidins, are antifungal against fungi belonging to the genera *Fusarium* and *Aspergillus* (Lauth *et al.* 2005; Zhang *et al.* 2009; Yang *et al.* 2011). They are also active against many yeasts and molds (Lauth *et al.* 2001). Parasites like *Trichomonas vaginalis, Trichodina, Cryptocaryon theront, Amyloodinium dinospore* and *Ichthyophthirius theront* also displayed vulnerability towards some of the piscidin isoforms (Colorni *et al*. 2008, Sung and Lee 2008; Sung *et al*. 2008). β-defensin characterized from orange spotted grouper and rainbow trout (Falco *et al.* 2008; Guo *et al.* 2012; Alvarez *et al*. 2013) piscidins and hepcidin from grouper and tilapia (Douglas *et al.* 2001; Wang *et al.* 2010; Rajanbabu and Chen 2011b; Pereiro *et al.* 2012) have exhibited promising antiviral activity against several fish viruses. Besides, they also exhibit anticancer activity against several cancer cell lines including HeLa, human breast cancer and fibrosarcoma cell lines (Chen *et al.* 2009a,b; Chang *et al.* 2011). Moreover *in vitro* inhibition of the proliferation of cancer cells by induced apoptosis in response to cytokine production like TNF-α, IL-10, IL-15 and IL-6 was also noticed (Chen *et al.* 2009b). Many fish AMPs are effective modulators of pro-inflammatory and other immune-related genes *in vivo* like IL-1β, IL-10, IL-22, IL-26, TNF-α, IFN-γ, NF-κB, lysozyme,

NOS2, MyD88, TLR4a, TLR1 and TLR3 (Pan *et al.* 2010; Neves *et al.* 2011; Meloni *et al.* 2015). Some fish AMPs have shown to possess chemotactic activity for leucocytes, monocytes, dendritic cells and T-lymphocytes also, which will in turn induce phagocytosis (Rohrl *et al.* 2010).

AMPs are expressed in almost all fish tissues and functional even in early embryonic development (Liang *et al.* 2013). Their role as premier artilleries of innate defense of fish is proved beyond doubt. However, functional characterization of various AMP isoforms should be carried out to elucidate possible physiological function of the peptide in host immune defense. Besides, before the peptide is brought into clinical trials several parameters like mode of action, efficiency, safety etc. have to be addressed (Li 2011). Peptides in large quantity are required for clinical trials. Recombinant expression in suitable expression hosts or chemical synthesis of the peptide is the sole possible way to overcome the hurdle. Both methods have merits as well as demerits. Heterologous expression in genetically modified *Escherichia coli* host strains is the cost effective and most widely used technique to obtain properly folded peptide in cost effective way (Li 2009). But, possibility of imminent toxicity of the expressed peptide to the host is one problem that has negative impact on recombinant expression (Li 2011). Another issue is the inefficiency of *E. coli* strain to use certain codons that are present in peptide to be expressed but rarely used in *E. coli*. All these parameters will affect the efficiency of recombinant expression (Li 2011; Haidong *et al.* 2013). Chemical synthesis of peptide is a costly affair. Moreover, *in vitro* disulphide bond formation is a pretty complex task to achieve (Klüver *et al.*

2006; Wu *et al*. 2003). Therefore, synthesis of peptides with multiple disulphide bonds such as defensins and hepcidins by solution or solid phase chemical synthesis is a tremendous as well as highly expensive affair. But it is the only way for functional characterization of toxic peptides. Besides, peptide modifications like isotype labelling, amino acid alterations and fusion peptides are possible only with chemical synthesis. Fish AMPs have been characterized functionally by both means were found to be efficient as antimicrobial agents (Moon *et al.* 2007; Cai *et al.* 2012; Haidong *et al.* 2013; Li *et al.* 2014; Zhang *et al.* 2014; Janakiraman *et al.* 2015; Zhao *et al.* 2015). Biological activity of fish AMPs are similar to previously reported AMPs from various other sources.

1.6 Significance of the study

Microbial resistance to conventional antibiotics is increasing in an alarming pace due to illegitimate and indiscriminate usage of antibiotics. Doubling of the number of selectively evolved multiple drug resistant microbes day by day is a serious global public health issue. Invention of therapeutic agents with potential to overcome bacterial resistance is a subject to be addressed immediately. AMPs are endogenous antibiotics produced by all living organisms ranging from most primitive archaea to modern human beings. They are the effector molecules of innate immune system of the host and provide fast and effective means of defense against invading pathogens. AMPs could neutralize wide range of microbes effectively and rapidly without giving chance to develop microbial resistance. This potential to overcome bacterial resistance as well as

synergism with conventional antibiotics makes them promising candidates as novel therapeutic agents. Although more than 2000 AMPs have been reported from various sources, reports of AMPs from fishes are hardly below 10%.

Marine fishes still remain as an unexploited source of AMPs. Since marine ecosystem is a microbe dominated environment, organisms like fishes which are profoundly dependent on innate immunity may possess novel antimicrobial agents with specific as well as broad spectrum activity. The main objective of the present study is to bio-prospect for novel fish-derived AMPs with potential to develop into future therapeutics from marine fishes. Moreover, identification of new AMPs from fishes will benefit to better understand innate defense mechanisms of fish which will further aid in improvised health management practice in aquaculture.

1.7 Objectives of the study

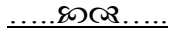
The specific objectives of the study are:

- Screening of antimicrobial peptides in marine fishes
- Molecular characterization and phylogenetic analysis of AMPs in marine fishes
- Heterologous expression of AMP and its functional characterization
- Structural and functional characterization of synthetic AMP

1.8 Outline of the thesis

The thesis is presented in seven chapters. Chapter 1 is the general introduction. Chapter 2 deals with the screening of marine fishes for novel

antimicrobial peptides, a gene based approach. Chapter 3 deals with molecular characterization and phylogenetic analysis of histone-derived antimicrobial peptides (Teleostin from *Cynoglossus semifasciatus* and *Tachysurus jella*). Chapter 4 describes Hepcidin from fishes *Chlorophthalmus bicornis* (Hepc-CB1) and *Zanclus cornutus* (Zc-Hepc1). Chapter 5 deals with the recombinant expression of a hepcidin isoform identified from the fish *Zanclus cornutus* (Zc-Hepc1) using pET32a+ expression vector system in genetically modified *E. coli* expression host (Rosetta-gami™ B (DE3) pLysS) and functional characterization of the peptide using antimicrobial assays. Chapter 6 deals with antibacterial, anticancer and cytotoxic property of synthetic hepcidin (Hepc-CB1 identified from the fish *C. bicornis*). The present study is summarized in Chapter 7 with special emphasis on salient findings of the study. This is followed by a list of References, GenBank accessions and Publications.



Chapter 2

SCREENING FOR ANTIMICROBIAL PEPTIDES IN MARINE FISHES-GENE BASED APPROACH

Contents

2.1 Introduction

Fishes, the first jawed vertebrates represent the transition point in the phylogenetic spectrum between organisms which profoundly depend on adaptive immunity (higher vertebrates) and those which solely rely on innate immunity (invertebrates) (Magnadottir 2006). Though fishes possess adaptive immunity, it is feeble and short lived, which make them hooked on to the innate immune parameters (Workenhe *et al.* 2010). In fishes, external surfaces like skin, gill and intestinal surfaces are the doorways of potential harmful opportunistic pathogens. The integument and integumental secretions of fish such as mucus are multifunctional in role and armed with a repertoire of humoral (complement, transferrins, anti-proteases, haemolysin, lysozyme, interferon, C-reactive protein, AMPs etc.) and cellular (leukocytes, including macrophages, neutrophils, lymphocytes and scavenger endothelial cells) innate defense parameters, which interact to initiate the inflammatory response (Whyte 2007; Workenhe *et al.* 2010).

Antimicrobial peptides are key effector molecules of innate host defense virtually present in all forms of life (Zasloff 2002). Accumulating evidences indisputably prove the inevitable role of AMPs in innate immune defense of fishes. Some AMPs are widely distributed among fishes, whereas some are specific to certain groups (Douglas *et al.* 2001; Shi and Camus 2006). They are synthesized and circulated in most of the tissues besides delicate external surfaces like gills, skin and intestine, that are in constant contact with dynamic and challenging external environment (Fernandes *et al.* 2010). Pardaxin, a cytotoxic peptide identified from *Pardachirus marmoratus,* is the first identified and characterized AMP from fish. Since then, more than hundred different AMPs belonging to several groups have been identified; of which some show wide distribution and diversity among various fishes, while some others are specific to certain families or orders of fishes (Masso-Silva and Diamond 2014). Defensins, cathelicidins, piscidins, pleurocidins, hepcidins and histone-derived peptides are the major groups of fish AMPs reported (Rajanbabu and Chen 2011b; Masso-Silva and Diamond 2014). Except histone derived peptides, all other classes of peptides are produced as an inactive pre-propeptide with a leader signal peptide for cellular translocation, a mature bioactive peptide and a charge neutralizing prodomain before (hepcidin, defensin, cathelicidin) (Zanetti *et al.* 1995; Singh *et al.* 2011) or after the mature peptide (piscidins) (Noga and Silphaduang 2003).

a) Defensins

Defensins are cysteine and arginine rich peptides of less than 100 amino acids, stabilized by 6-8 disulphide bonds. They are widely distributed among

living organisms and produced by both plants and animals (Andreu and Rivas 1998). Plant defensins contain 8 cysteines, forming four intramolecular disulphide bonds whereas insect and vertebrate defensins are structured by three intra-molecular disulphide bonds formed by six cysteine residues. Based on the structural arrangement like positioning of disulphide bonds, vertebrate defensins could be classified into three groups (Zou *et al*. 2007). They are α, β and Θ defensins. In α-defensins, disulphide bonds are formed by the linking between C1–C6, C2–C4 and C3–C5, whilst in β-defensins, C1–C5, C2–C4, and C3–C6 cysteines link to form disulphide bridges. Alpha and beta defensins are more common in occurrence whereas Θ-defensin is reported only from certain primates. The defensins identified from fishes solely belonged to β-defensins (Tran *et al*. 2002). They are synthesized in many cell types including lymphocytes, monocytes, macrophages, neutrophils, epithelial cells, keratinocytes etc. (Lehler *et al*. 1993; Ganz 1994; Martin *et al*. 1995). They showed antimicrobial activity at micro-molar levels against a wide range of Gram-positive and Gram-negative bacteria, fungi and some enveloped viruses (Falco *et al*. 2008; Chia *et al*. 2010). In addition to antimicrobial activity, they also possess immunomodulatory and chemotactic properties. Existence of multiple copies of defensins among vertebrates suggest expansion and evolution of defensin in vertebrates, probably from a common ancestor by gene or genome duplication events (Zou *et al.* 2007). Zou and co-workers reported multiple β-defensin-like peptides from teleost fish species including zebrafish *Danio rerio,* Puffer fish *Takifugu rubripes* and *Tetraodon nigroviridis,* in 2007 for the first time. Later defensin-like peptides have been identified from a number of fishes, including Medaka, *Oryzias latipes*

(Zhao *et al.* 2009), Rainbow trout, *Oncorhynchus mykiss* (Casadei *et al.* 2009), Olive flounder, *Paralichthys olivaceus* (Nam *et al.* 2010), Gilthead sea bream, *Sparus aurata* (Cuesta *et al.* 2011), Mandarin fish, *Siniperca chuatsi* (Wang *et al.* 2012b), Common cultured carp, *Cyprinus carpio* (Marel *et al.* 2012) and Orange-spotted grouper, *Epinephelus coioides* (Guo *et al.* 2012). These results suggest that defensins are inexorably involved in host immune responses to invasion of pathogens, and open new avenues for the design of antimicrobial agents in aquaculture and medicine.

b) Cathelicidins

Cathelicidins are the family of antimicrobial peptides with a highly conserved N-terminal sequence, which shows sequence similarity to the members of cystatin superfamily of cysteine proteinase inhibitors called cathelin, hence the name cathelicidins (Zanetti *et al.* 1995). This family of peptides was first characterized by Zanetti *et al* (1995). Cathelicidins are a highly diverse group of peptides and exhibit utmost diversity in amino acid composition as well as secondary structure of mature peptide region. Depending upon the nature of the variable C-terminus, cathelin-associated peptides could be grouped into three. They are, (1) those that have a high content of one or two amino acids, (bactenecin, indolecithin) often proline/arginine/phenylalanine; (2) those that contain intra-molecular disulphide bonds (protegrin) and (3) those with amphiphilic regions to form α-helices (Papagianni 2003). The gene coding for cathelin-associated peptide contains four exons, the first three of which code for the conserved signal peptide and the cathelin proregion, whereas the fourth exon codes for processing sites and the variable C-terminal antimicrobial domain that

is cleaved off by the enzyme elastase/furin upon formation of the mature antimicrobial peptide (Zhao *et al*. 1995a,b; Gudmundsson *et al*. 1995, 1996). Cathelicidins are well established and thoroughly characterized in mammals; nevertheless, the first non-mammalian cathelicidin was identified from the Atlantic hag fish, *Myxine glutinosa* (Uzzell *et al.* 2003).

Cathelicidin-like peptides are identified mostly from salmonids including, (*Salmo trutta fario*), brook trout (*Salvelinus fontinalis*), grayling (*Thymallus thymallus*) (Scocchi *et al.* 2009), two genes from Atlantic salmon, *Salmo salar* and Rainbow trout (*Onchorhynchus mykiss*) (Chang *et al.* 2005), 3 cathelicidin isoforms from Atlantic cod (*Gadus morhua*) and a third exon deleted modified isoform from Arctic charr (*Salvelinus alpinus*) and Brook Trout (*S. fontinalis*) (Maier *et al.* 2008). Based upon the number of disulphide bonds, fish cathelicidins could be grouped possibly into two groups namely, CATH1 and CATH2. Peptides with two cysteines and a single disulphide bond will form the first group (CATH1) and peptides with more than one disulphide bond as CATH2. The second group is relatively shorter in size due to the deletion of one of the four exons of the gene, namely 3rd exon (Maier *et al.* 2008). Fully functional mature bioactive cathelicidins also had been extracted, purified and characterized from different tissues of *Gadus morhua* using HPLC (Broekman *et al.* 2011). Tissue specific expression profiling using RT-PCR indicated the presence of cathelicidin mRNA from the very early stages of larval development. The presence of cathelicidin mRNA at this early stage is likely due to the transfer of maternal RNA. All these factors point to one statement that cathelicidin is an important primary defense mechanism in many of the fishes especially salmonids.

c) Piscidins

Piscidins are cationic, amphipathic α-helical AMPs rich in phenyl alanine and histidine and are the most salt tolerant peptides identified till date (Noga and Silphaduang 2003; Chekmenev *et al*. 2006). First piscidin was identified from the mast cells of the commercially cultured hybrid striped bass (white bass, *Morone chrysops* - female x striped bass, *Morone saxatilis*- male) (Silphaduang and Noga 2001). Moronecidins (Lauth *et al.* 2001), chrysophsin (Iijima *et al.* 2003), dicentracin (Salerno *et al.* 2007), misgurins and pleurocidins (Douglas *et al*. 2001; Sun *et al.* 2007) are well-known broad spectrum AMPs belonging to the group pisicidins, the most common antimicrobial peptides of fishes. Apart from fishes, piscidin-like peptides have also been reported from spotted sea horse *Hippocampus huda* (GenBank ID: AY864343). Piscidins are generally synthesized as an inactive prepropeptide, structured as leader signal peptide, bioactive cationic mature peptide and a charge neutralizing prodomain (Valore and Ganz 1998). Mature piscidins are generally 22-26 aa in length except for a novel piscidin 4 isoform, which is 44 amino acid in length (Noga *et al.* 2009; Salger *et al.* 2011). Naturally they may exist as a mixture of amidated and non-amidated peptides and the C-terminal amidation helps to improve antimicrobial property (Jia *et al*. 2000; Falla *et al.* 1996; Chekmenev *et al*. 2006). Piscidins have broad spectrum activity against bacteria (Corrales *et al*. 2010; Pan *et al*. 2011a; Lee *et al*. 2012b), fungi (Sung *et al.* 2008), protozoans and parasites (Pan *et al*. 2010). Besides, they also possess immunomodulatory (Lee *et al*., 2012b; Masso-Silva and Diamond 2014; Pan *et al*. 2011a), anti-viral (Wang *et al.* 2010), anti-cancer (Lin *et al.* 2009) and anti-nociceptive properties (Chen *et al.* 2015a). The piscidin and epinecidin-

1 are suitable for treating and preventing infections caused by emerging antibiotic-resistant strains of bacteria in agriculture and fishery as they exhibit effective bacteriostatic and bactericidal activity against antibiotic-resistant bacteria (Pan *et al*., 2007, 2009). Potential applications of piscidin has grown up to the use of epinecidin gene incorporated or genetically modified *Artemia* as feed in aquaculture (Jheng *et al.* 2015).

d) Hepcidin

Hepcidin is an acute phase short cysteine-rich peptide concurrently functioning as iron regulatory hormone as well as antimicrobial peptide, probably evolved from an antimicrobial peptide gene (Shi and Camus 2006). The Solution NMR structure defined hepcidin as a β-hairpin. The mature hepcidin will contain a multiple cysteine core domain signature, usually 6-8 cysteines arranged in a pattern C1- C8, C2-C7, C3-C6 and C4-C5 (Lauth *et al.* 2005). Hepcidin was primarily identified from the human blood ultra-filtrate as liver expressed antimicrobial Peptide (LEAP-1) (Krause *et al.* 2000)/ hepatic antimicrobial peptide (HAMP) (Park *et al.* 2001). Hepcidin-like peptides are widely distributed among the members of subphylum Vertebrata with representatives in all classes including fishes. In fishes, HAMPs are transcription products of multiple genes of two main lineages *i.e*., HAMP1 and HAMP2. HAMP1-like peptides are distributed in all groups of fishes and is an orthologue of mammalian HAMP while HAMP2-like group is reported only from acanthopterygian fishes (Hilton and Lambert 2008). These two groups of peptides differ mainly in the overall cationicity and the presence or absence of the presumed iron binding motif QSHLS/DTHFP. In fishes, though the expression of hepcidin genes could be detected in all tissues, liver is presumed to be the

major site of hepcidin synthesis (Solstad *et al.* 2008; Singh *et al.* 2011). In fishes, hepcidin was identified first time from hybrid striped bass (Shike *et al.* 2002), later a number of hepcidin-like peptides were reported from Tilapia (Huang *et al.* 2007), Black porgy (Yang *et al.* 2011), Sole fish (Wang *et al.* 2012c), Channel cat fish (Tao *et al.* 2014), Turbot (Pereiro *et al.* 2012), Yellow croaker (Zhang *et al.* 2009), Medaka (Cai *et al.* 2012), Mud loach (Nam *et al.* 2011), Pacific mutton Hamlet (Masso-Silva *et al.* 2011) and Snake head (Gong *et al.* 2014) .

e) Histone-Derived AMPs

Histones are classical DNA binding proteins. However several studies have proved that they have a secondary role, which is to aid in immune response of an organism by acting as proteins/peptides with antimicrobial activity (Kawasaki *et al.* 2003; Smith *et al*. 2010). First report on the antimicrobial property of histones was from calf thymus. There are two main type of histones; core histones (H2A, H2B, H3 and H4) and linker histone (H1), rich in either lysine or arginine residues. Antimicrobial property has been reported in all the five histone proteins. Additionally, several naturally occurring AMPs share sequence identity with portions of various histone subunits, implying that both whole and fragmented histone proteins may possess antimicrobial properties (Kawasaki *et al.* 2008). Though histone derived antimicrobial peptides (HDAPs) are reported from all five classes of histone proteins, H2A derived AMPs lead the chart in terms of numbers. Parasin (Park *et al.* 1998b), hipposin (Birkemo *et al.* 2003), buforin I and II, abihsin (Zoysa *et al.* 2009), himanturin (Sathyan *et al.* 2012a), harriottin (Sathyan *et al.* 2013) sunettin (Sathyan *et al.* 2012c) and molluskin (Sathyan *et al.* 2012b), represent

histone H2A derived AMPs while onchorhyncin II (Fernandes *et al*. 2004), SAM (Salmon antimicrobial protein) (Richards *et al.* 2001) are derivatives of histone H1. Antimicrobial properties of histone (Kawasaki *et al.* 2008; Seo *et al.* 2011), H3 and H4 are also well documented (Tagai *et al.* 2011a).

Marine ecosystem is the largest and unique habitat that harbours more than two third of earths biodiversity. Fauna of marine ecosystem consist mainly of invertebrates and lower vertebrates. Fishes contribute a significant share to the total biodiversity of marine ecosystem and could be considered as prospective source of antimicrobial peptides as they mainly rely on humoral primary immune defense mechanism to thrive in the highly dynamic and challenging external environment rich with microorganisms. However, though a number of AMPs have been identified from variety of cultured fishes, marine/wild fishes still remain an underexplored source of AMPs probably due to the inaccessibility of the habitat. The present chapter deals with the screening of both commercial as well as non-commercial wild fishes belonging to different strata of marine ecosystem for the presence of major groups of fish AMPs.

2.2 Materials and Methods

2.2.1 Sample Collection

Samples were collected from diverse habitats of marine ecosystem, beginning from estuaries and near shore waters to deep waters upto a depth of 500 to 1000 m. Estuarine samples were collected from various parts of Vembanad estuarine system and Cochin back waters with the help of fisher folk. Operators of Chinese dip nets of Fort Kochi and Vypeen and small 'odam fishers' of Kalamukku harbour, Kochi, Kerala, India, also contributed

to the estuarine sample collection. Coastal samples were obtained from the daily fishing cruises of FORV *Sagar Sakthi*, CIFT Kochi. Deep sea sampling (200-1000 m) was carried out on board FORV *Sagar Sampada*, CMLRE, MoES. Samples were obtained from four cruises (Cr no 258, 262, 291 and 292), covering the East and West coast of India as well as Andaman Sea.

2.2.2 Pre Requirements

Since the proposed work mainly depended on expressed mRNAs of the peptides, obtaining high quality RNA is an obligatory prerequisite. RNA is highly sensitive to Ribonucleases (RNases). Hence certain basic precautions were taken to obtain high quality RNA. All materials/utensils used for RNA isolation including glasswares, homogenizers, scissors, forceps and gloves were treated with 0.1% Diethyl pyro carbonate (DEPC) to make it free of RNases. The treatment process involved overnight incubation of glassware and solution with DEPC water at room temperature followed by 1 h autoclaving at 15 lbs to remove the dissolved DEPC as ethanol and CO_2. Another autoclaving was also performed at 121 lbs for 15 min to make the utensils and solutions sterile. All the chemicals used in the RNA isolation procedure were free of RNases. Anticoagulant used was prepared in RNase free water using molecular grade chemicals.

2.2.3 Tissue Processing

Tissue samples from gills, liver, kidney etc. were collected from the live/freshly caught dead fish (Fish samples collected from 500 m depth zone of the sea will die before reaching the deck of the vessel due to the pressure difference). Tissues like blood, gill, head kidney, liver, skin, muscle and intestine were analysed as the propensity of occurrence of AMP is high in

these tissues. Blood was collected from the caudal sinus/lamellar artery near the gill region/directly from heart with syringe rinsed with pre-cooled RNase free anticoagulant (RNase free, 10% sodium citrate, pH 7.0). Approximately 1ml of blood was then transferred to 1ml of TRI™ reagent. Rest of the tissues (gills, head kidney, liver, skin, muscle and intestine) were carefully dissected out immediately after sacrificing the fish humanely and transferred to TRI™ reagent. Samples preserved in TRI™ reagent were stored at -80 °C until use.

2.2.4 RNA Isolation

Total RNA was isolated from the tissue sample preserved in TRI™ reagent according to the manufacturer's protocol (Sigma, USA). In brief, tissues (about 1 ml blood or 50 mg of gill/other tissues) were homogenized in 1 ml TRI Reagent with a tissue homogenizer and allowed to stand for 5 min at room temperature to ensure complete dissociation of the nucleoprotein complexes. The homogenate was then centrifuged at 12,000 x g for 10 min at 4 °C to remove cell debris and other insoluble materials and supernatant was transferred to fresh RNase free 1.5 ml vials. To the homogenate 0.2 ml chloroform per 1 ml TRI reagent was added, shaken vigorously for 15 sec and allowed to stand for 2-15 min at room temperature. Again centrifuged at 12,000 x g for 15 min at 4 °C to get the mixture separated into three phases; a red organic phase (containing protein), an interphase (containing DNA) and a colourless upper aqueous phase (containing RNA). The aqueous phase was then transferred to a fresh 1.5 ml vial, 0.5 ml isopropanol per ml TRI Reagent was added and allowed to stand for 5-10 min at room temperature. Another centrifugation at 12,000 x g for 10 min at 4 °C was performed to precipitate RNA and the RNA thus precipitated

on the side and bottom of the tube was washed twice by adding 1 ml ice cold ethanol (75%). The samples were then vortexed and centrifuged at 7500 x g for 5 min at 4 °C. RNA pellets were air dried for 5-10 min and dissolved in RNase free water at 55-60 °C for 10-15 min.

2.2.5 Determination of the quantity and quality of RNA

The quality of the RNA is analysed by agarose gel electrophoresis. Quantity and purity of RNA was determined using a UV Spectrophotometer (U-2900, Hitachi) by measuring optical density (OD) at 260 and 280 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of an RNA preparation and the pure RNA will be having an absorbance ratio (A_{260}:A_{280}) ≥1.8. Only those RNA preparations which are having an absorbance ratio ≥1.8 were used for cDNA synthesis. Absorbance maximum for RNA is at 260 nm, hence absorbance at 260 nm was taken for quantification and quality assessment of RNA and quantified as follows.

1 OD at 260 nm is equivalent to about 40 µg/ml of RNA

∴ RNA concentration (µg/ml) = OD at 260 nm × Dilution factor × 40

2.2.6 cDNA synthesis

From the total RNA preparation, mRNAs were converted to single stranded complementary DNA (cDNA) by Reverse transcription using oligo dT primers. The reaction was carried out in a 20 µl reaction mixture containing 5 µg total RNA, 1x RT buffer, 2 µM dNTP, 2 µM oligo d(T_{20}), 20 U of RNase inhibitor and 100 U of MMLV Reverse transcriptase (Fermentas, Inc.). The thermal cycle was 42 °C for 1 h followed by an

inactivation step at 85 °C for 15 min and the cDNA synthesized was stored at -20 °C until use.

2.2.7 PCR amplification

Primarily PCR amplification for reference gene, β-actin was carried out using gene specific primers for fish β-actin to test the quality of mRNA (Table 2.1). Later the cDNAs were screened for the specific group of antimicrobial peptides, like piscidins, cathelicidins, hepcidins, histone derived peptides etc. with corresponding gene specific AMP primers (Table 2.1).

Table 2.1: Primers used in the study to amplify β-actin and AMP specific genes

Gene	Primer Sequences	Annealing temperature	Reference
β-actin	F:gatcatgttcgagaccttcaacac R:cgatggtgatgacctgtccgtc	60 °C	Hassanin *et al*. 2009
Epinecidin	F:cgctctttcttgtgttgtcg R:tgcgtaacacagattccaga	60 °C	(designed in the present study)
Pleurocidin	F:aagcccactttgtattcgca R:gctgttgcaggtaaaagtctga	60 °C	(designed primer)
Pleurocidin II	F:gcccactttgtattcgcaag R:ctgaaggctccttcaaggcg	55 °C	(designed primer)
Dicentracin	F:ctttcttgtgctgtcgatgg R:ctgctctttcagatgaaccg	55 °C	(designed primer)
Hepcidin	F:cagacaggagaagaagtcaaaggag R:attatgacaatacacagtaaaatctgc	60 °C	(Ren *et al*. 2006)
Hepcidin II	F:gaacctgcagcagacaccacatccg R:cgaagcagtcaaaccctcctaagatg	55 °C	(designed primer)
Hipposin	F:atgtccggrmgmggsaarac R:gggatgatgcgmgtcttcttgtt	60 °C	(Birkemo *et al.* 2003)
Parasin	F:atgggaacgtcttcaacctcc R:ttagggatattcgatgattt	50 °C	(Park *et al.* 1998b)
Cathelicidin	F:catcctgctcgctgtggctgtcct R:gcgatttccatcactgtgatctct	60 °C	(Anderson and Yu 2003)

The PCR amplification of cDNAs were performed in a 25 µl reaction volume containing 1X standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The PCR condition involved an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, 68 °C for 30 s and a final extension at 68 °C for 10 min. Annealing temperature varied with different primers as shown in Table 2.2. PCR products were stored at 4 °C until use.

2.2.8 Analysis of PCR product and agarose gel electrophoresis

PCR products were analyzed on 1.5% agarose gel prepared in 1x TBE buffer (Tris-base- 10.8 g, 0.5 M EDTA– 4 ml, Boric acid– 5.5 g, double distilled water– 100 ml, pH– 8.0). For that, 1.5 g agarose was measured out and melted in 1x TBE buffer (100 ml).Two microliter ethidium bromide (1 mg/ml stock stored in dark) was added and poured into gel casting trays with pre-set combs. The tray with solidified agarose, gel was then immersed in a buffer tank filled with 1x TBE buffer. PCR product (10 µl) was mixed with 2 µl of 6x gel loading dye (1% bromophenol blue– 250 µl, 1% xylene cyanol– 250 µl, glycerol– 300 µl, double distilled water– 200 µl) and loaded into the wells. Electrophoresis was done at a voltage of 3-5 volt/cm till the indicator dye (bromophenol blue) front migrated to three fourth of the gel. The gel was visualized on a UV Trans-illuminator using the Gel Doc XR system and documented using Quantity One software (Bio-Rad Hercules, Ca).

2.2.9 Sequencing

The PCR products were purified and sequenced using ABI Prism Sequencing Ready Reaction kit (BigDye Terminator Cycle) on an ABI

Prism 377 DNA sequencer at SciGenom Sequencing Facility, Kakkanad, Kochi, Kerala.

2.2.10 Sequence Analysis and Molecular Characterization

The sequences were analyzed, trimmed and assembled using GeneTool software. The cDNA-based gene sequences were translated using Expert Protein Analysis System (http://au.expasy.org/). Homology searches of nucleotide sequence as well as the deduced amino acid sequences were performed using BLASTn and BLASTp algorithm of the National Centre for Biotechnological Information (http://www.ncbi.nlm.nih.gov/blast). Pre-deposited sequences of required AMPs were retrieved from NCBI and multi-aligned using ClustalW and GeneDoc computer programmes. Physico-chemical properties and peptide characteristics were analyzed using ProtParam Tool (http://cn.expasy.org/cgi-bin/protparam), antimicrobial peptide data base (APD) (http://aps.unmc.edu/AP/main.php), Protein Calculator v 3.3 (http://protcalc.sourceforge.net/), PepDraw (http://www.tulane.edu/~biochem/) and Protean module of the DNASTAR Lasergene sequence analysis software suite. Enzyme cleavage sites, processing sites of signal peptide and the propeptide convertase were identified using the Peptide Cutter tool (http://web.expasy.org/peptide_cutter/), SignalP program (http://www.au. ExPASy.org/) and ProP 1.0 server (http://www. cbs.dtu.dk/services/ProP/) respectively. PDB data was generated SWISS-MODEL, a homology based protein modelling server. Using the PDB data thus generated, secondary and spatial structures were generated with the softwares PyMOL and ViewerLite version 4.2. Amphipathic characterization and helical properties of the peptide were

delineated with Schiffer Edmundson helical wheel modelling using the software DNASTAR Lasergene 10.1.

2.2.11 Phylogenetic analysis

Amino Acid sequences of respective AMP genes were retrieved from GenBank and multi-aligned using ClustalW and GeneDoc computer programmes. Phylogenetic analysis was carried out using MEGA version 5.05. Phylogenetic tree was constructed by neighbour joining (NJ) method with 1,000 repetitions of bootstrap.

2.2.12 Tissue wise expression of Epinecidin

For tissue specific expression analysis of epinecidin, six tissues (gills, intestine, liver, head kidney, muscle and skin) samples were collected from the fish, total RNA was extracted and converted to cDNA. Tissue specific expression of epinecidin was determined by semi-quantitative RT-PCR analysis using β-actin as the internal control. The template cDNA was diluted and adjusted as 5 µg/µl. PCR amplification of 1 µl of the diluted cDNA was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The PCR condition involved an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, 68 °C for 30 s and a final extension at 68 °C for 10 min. PCR product was analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. The intensity of the gel bands were measured using ImageJ analysis software.

2.2.13 Molecular identification of fish

Taxonomic identities of ambiguous species were confirmed by molecular tools. DNA barcoding was carried out and mitochondrial cytochrome oxidase subunit 1 gene was used as the barcoding region.

Genomic DNA was isolated from the gills of fishes of unknown identity for molecular barcoding using TRI® Reagent according to manufacturer's protocol with slight modification. Briefly, approximately 50 mg of tissue was homogenized with 1 ml in a tissue homogenizer. The homogenate was transferred to 1.5 ml microcentrifuge tubes (MCTs). To remove high content of protein, fat and extracellular materials such as muscles and fat tissue, the homogenate was centrifuged at 12,000 x *g* for 10 minutes at 2-8 ^{o}C and allowed to stand for 5 minutes at room temperature. The supernatant was then transferred to fresh vial, added 0.2 ml chloroform, shaken vigorously for 15 seconds and allowed to stand at room temperature for 15 minutes. It was then centrifuged at 12,000 x g for 15 minutes at 4°C so as to separate the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA) and a colorless upper aqueous phase (containing RNA). The aqueous phase overlaying the interphase was carefully removed and DNA was precipitated from the interphase and organic phase by adding 0.3 ml of 100% ethanol per 1 ml of TRI® reagent used in sample preparation. Mixed gently by inversion to evade DNA shearing and allowed to stand for 2–3 minutes at room temperature. Another centrifugation at 2,000 x g for 5 minutes at 4 °C was performed to get DNA pellets and the supernatant was discarded. The DNA pellet was then washed twice in 1 ml of 0.1 M

trisodium citrate, 10% ethanol solution. During each washing step, the DNA pellet was allowed to stand for at least 30 minutes with occasional mixing to remove phenol completely from DNA. Subsequently centrifuged at 2,000 x g for 5 minutes at 4 °C and DNA pellet was re-suspended in 75% ethanol (1 ml), allowed to stand for 10–20 minutes at room temperature followed by centrifugation at 2,000 x g for 5 minutes at 4 °C. Then the DNA pellet was dried for about 30 min at room temperature and dissolved in PCR grade DNase free water with repeated slow pipetting with a micropipette. Centrifuged at 12,000 x g for 10 minutes at room temperature to remove any insoluble material and transferred the supernatant to a fresh vial.

The quality of the DNA was analysed by agarose gel electrophoresis. Quantity and purity of DNA was determined using a UV Spectrophotometer (U-2900, Hitachi) by measuring the optical density (OD) at 260 nm and 280 nm. The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of the DNA preparation and the pure DNA will be having an A260:A280 ≥1.8. Only those DNA preparations which is having an absorbance ratio (A260:A280) ≥1.8 were used for cDNA synthesis. Absorbance maximum for DNA is at 260 nm, hence absorbance at 260 nm was taken for quantification and quality assessment of DNA and quantified as follows.

1 OD at 260 nm is equivalent to about 50 µg/ml of DNA

∴ DNA concentration (µg/ml) = OD at 260 nm × Dilution factor × 50

The PCR reaction was carried out in 25 µl reaction mixture containing target 1 µl DNA (100 ng/µl), 1x standard Taq buffer (10 mM Tris–HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The PCR reaction was analyzed on 0.8% agarose gel, PCR products were purified and sequenced at SciGenom Sequencing Facility, Kakkanad, Kochi, Kerala, using ABI Prism Sequencing Ready Reaction kit (BigDye Terminator Cycle) on an ABI Prism 377 DNA sequencer.

2.3 Results

2.3.1 Fish AMPs detected in the study

AMPs, belonging to the group piscidins, histone-derived peptides and hepcidins could be identified. A piscidin called epinecidin from orange spotted grouper (*Epinephelus coioides*), histone derived peptides from marine catfish (*Tachysurus jella*) and Bengal tongue-sole, (*Cynoglossus semifasciatus*) (chapter 3), hepcidin isoforms from Spiny-jaw green-eye, *Chlorophthalmus bicornis* and Moorish idol, *Zanclus cornutus* (chapter 4) have been identified (Table 2.2). Out of these AMPs, the epinecidin detected from orange spotted grouper (*Epinephelus coioides*) alone is dealt with in this chapter. Histones and hepcidins are described in Chapter 3 and 4 respectively.

Table 2.2: Fish AMPs detected in the present study through gene based approach

Sl. No	Name of the Fish	AMP Identified	Amplicon Size in bp
1	Orange spotted grouper (*Epinephelus coioides*)	Piscidin	495
2	Bengal Tongue-sole (*Cynoglossus semifascitus*)	Histone derived peptide	245
3	Marine Cafish (*Tachysurus jella*)	Histone derived peptide	245
4	Spinyjaw greeneye (*Chlorophthalmus bicornis*)	Hepcidin	294
5	Moorish Idol (*Zanclus cornutus*)	Hepcidin	267

2.3.2 Molecular characterization of Epinecidin

A 495 bp amplicon was obtained from the cDNA of orange spotted grouper *Epinephelus coioides* (Fig. 2.1) using primers specific to the AMP Epinecidin-1 (Fig. 2.2).

Fig. 2.1: Orange spotted grouper, *Epinephelus coioides*

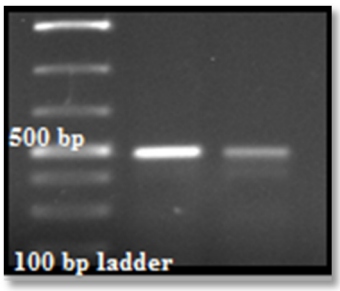


Fig. 2.2: 495 bp amplicon obtained with specific primer epinecidin

2.3.2.1 Sequence analysis and peptide characterization

An open reading frame of 201 nucleotides encoding a 67 amino acid (aa) prepropeptide could be identified from the full length cDNA (Fig. 2.3) Blast analysis of the nucleotide and deduced amino acid sequence revealed that the peptide belonged to the group piscidins. Nucleotide sequences of the peptide exhibited 98% similarity to epinecidin-1 identified from *Epinephelus coioides* and 97% similar to piscidin 2 identified from *E. malabaricus*, whereas the deduced amino acid sequence exhibited 100% similarity to both the epinecidn 1 and piscidin 2. Though the amino acid sequences showed 100% similarity, nucleotide sequence of the open reading frame (ORF) exhibited 99% similarity only. The prepropeptide was of 67 aa in length with 22 aa signal peptide, 25 aa mature peptide and a 20 aa C-terminal prodomain. SignalP software has identified a cutting site for the enzyme signal peptidases at the N-terminus of the peptide, between 22nd glycine and 23rd phenylalanine. Presence of the motif RX (K/R) H, which is typical for the processing of propeptide convertase, ensures another cleavage between 47th histidine and 48th glycine resulting in the formation of a 22 aa leader signal peptide a 25 aa mature peptide and 20 aa prodomain. The mature epinecidin is highly cationic and is having a net charge of +5 owing to the presence of 9 basic amino acids. The peptide is found to have a predicted molecular weight of 2.95 kDa, a hydrophobic index of 48% and an estimated isoelectric point (p*I*) of 12.31. The 67 aa prepropeptide is termed as pro-epinecidin and the mature bioactive peptide as epinecidin. Both the nucleotide and deduced amino acid sequences of epinecidin identified from *E. coioides* have been submitted to GenBank (ID: KX090373).

ClustalW multiple alignments of proepinecidin and previously reported piscidins demonstrated that signal peptide region is highly conserved among piscidins. Apart from that, mature peptide as well as prodomain showed remarkable variation in amino acid which is evident on sequence alignment (Fig. 2.4). However, various piscidin-like peptides characterized from the genus *Epinephelus* showed high degree of conservation in all the three regions (Fig. 2.5). However based on amino acid substitution in the mature peptide region, piscidins of the genus *Epinephelus* could be categorized into four main types. Type 1 with amino acid sequence 'FIF' at N terminus and RRRH at C-terminus of the Mature peptide, type 2 with amino acid sequence 'FFF' at N-terminus and RRRHRH at the C-terminus, type 3 with amino acid FGL at the N-terminus and RRGEEQ at the C-terminus and is slightly different from these two groups and type 4 is entirely different from all the three types and is exceptionally long (Fig. 2.5).

```
ttggccctcttcagtcacagcttttgacattcacgctgagtcactggaaagaggatgagg
  W  P  S  S  V  T  A  F  -  H  S  R  -  V  T  G  K  R  M  R
tgcatcgccctctttcttgtgttgtcgctggtggtcctcatggctgaacccggggagggt
 C  I  A  L  F  L  V  L  S  L  V  V  L  M  A  E  P  G  E  G
tttatcttccacatcatcaaaggactctttcacgctggcaagatgatccatggacttgtc
 F  I  F  H  I  I  K  G  L  F  H  A  G  K  M  I  H  G  L  V
accaggagacgacatggcgtggaagagctgcaagacctggaccaacgtgcctttgaacga
 T  R  R  R  H  G  V  E  E  L  Q  D  L  D  Q  R  A  F  E  R
gagaaagcttttgcctgagtctacgatggtccatgtgaaagagccactctttgcttcaga
 E  K  A  F  A  -  V  Y  D  G  P  C  E  R  A  T  L  C  F  R
tggggaaaaaaatatatatattgctgttgaatataattataaaaaaaaaaaaaaaaaaaa
 W  G  K  K  Y  I  Y  C  C  -  I  -  L  -  K  K  K  K  K  K
agaaagaagaaaactggctcacgtgggtaaccattgcaaaatatttcacactgatctaat
 R  K  K  K  T  G  S  R  G  -  P  L  Q  N  I  S  H  -  S  N
tgatttttttggaagaaaacaaaaagtcagtgatttgaaataaatctggaatctgtgtta
 -  F  F  W  K  K  T  K  S  Q  -  F  E  I  N  L  E  S  V  L
cgcaaaagcaaaaat
 R K S K N
```

Fig. 2.3: Nucleotide and deduced amino acid sequence of Epinecidin identified from *Epinephelus coioides*. The proepinecidin is highlighted in three colours, Yellow- signal peptide, Green- Mature peptide and Grey- prodomain.

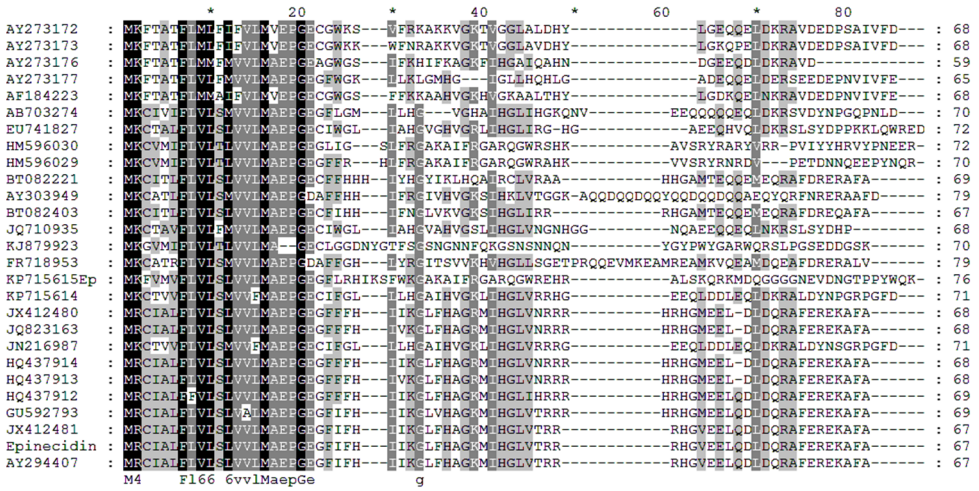


Fig. 2.4: ClustalW multiple alignment of epinecidin with previously reported piscidins and allied peptides reported from various fishes showing sequence similarity obtained using GeneDoc programme version 2.7.0. The four level colour coding indicates different degrees of conservation. Black background and white letters correspond to 100% conservation, gray background and white letters correspond to 80% conservation, and gray background and black letters correspond to 60% conservation.

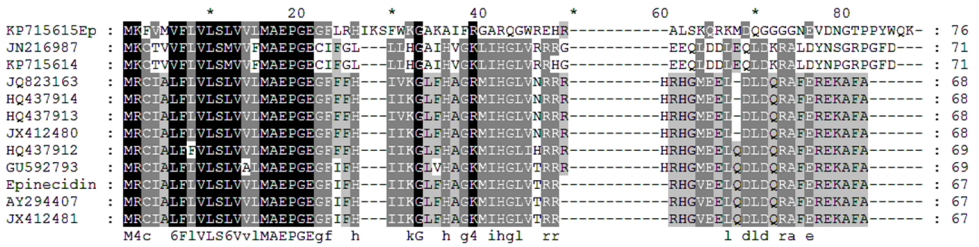


Fig. 2.5: ClustalW multiple alignment of epinecidin with piscidins reported from the genus *Epinephelus* showing sequence similarity

2.3.2.2 Phylogenetic Analysis

To delineate the evolutionary history and distribution of piscidins among teleost fishes, including dicentracin, moronecidin, pleurocidins and epinecidins phylogenetic analysis was carried out. Similarly sequence similarity and clustering pattern of piscidins was also analysed within the genus *Epinephelus*. Phylogenetic analysis of piscidin and allied peptides

clustered into two main lineages, pleurocidins and piscidin-like peptides (Fig. 2.6). Cluster formed of piscidin-like peptide is sub divided into 5 sub-clusters, Sub cluster 1 formed exclusively of various piscidins identified from *Epinephelus* sp. Sub-cluster 2 and 3 are formed of dicentracin like peptides identified from fishes of Scorpaenidae and Chanichthydae. Sub-cluster 4 is structured of Serranid and Sciaenid piscidins, sub cluster 5 is of relatively longer piscidins of 70-74 aa belonging to three families Serranidae, Sciaenidae and Moronidae. Cluster 6 is figured solely of pleurocidin-like peptides identified from the family Pleuronectidae. In the case of phylogentic tree of piscidins exclusively of the genus *Epinephelus*, piscidin 4 remains separate as an outgroup and rest of the piscidins formed a a single major cluster (Fig. 2.7) with three sub clusters formed of type 1, 2 and 3 piscidins depending upon the amino acid similarities and differences.

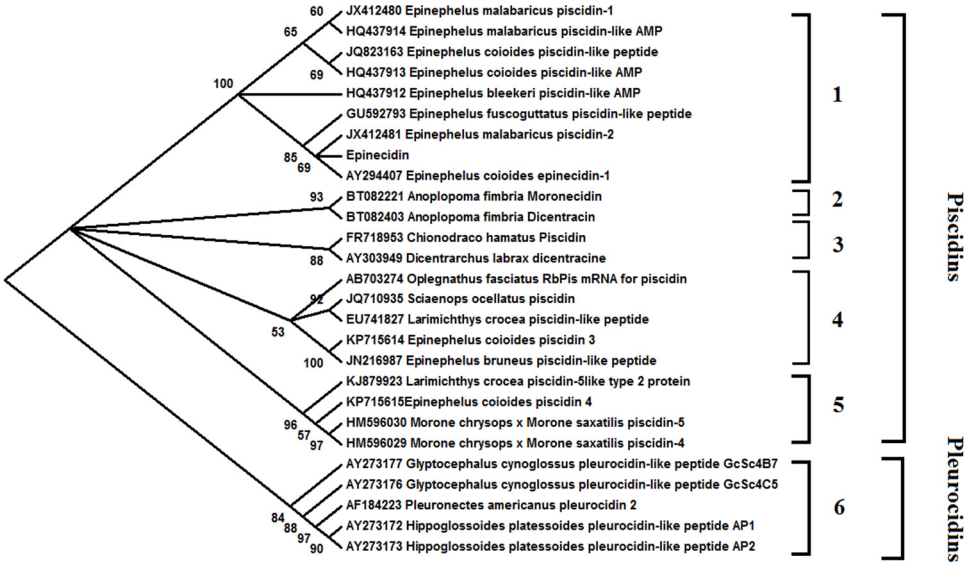


Fig. 2.6: A bootstrapped neighbor-joining tree obtained using MEGA version 5.0 demonstrating relationships between the amino acid sequences of epinecidin with other reported piscidins and allied peptides

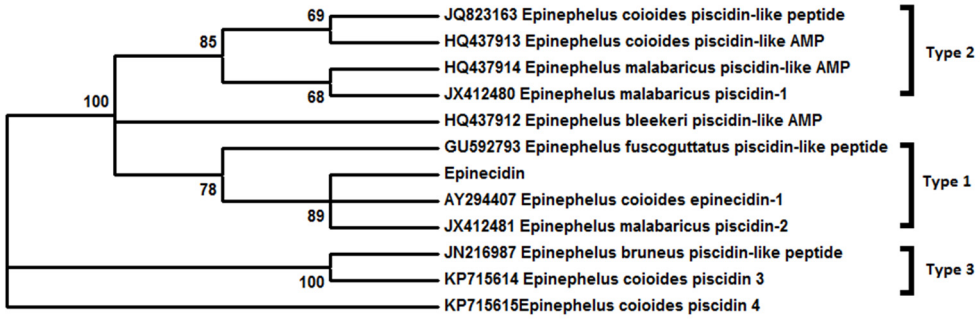


Fig 2.7: A bootstrapped neighbor-joining tree obtained using MEGA version 5.0 explaining relationships between the amino acid sequences of epinecidin and piscidins like peptides identified from the genus *Epinephelus*.

2.3.2.3 Tissue specific expression of Epinecidin

Gel image was analyzed using ImageJ software. The reverse transcription reaction showed uniform expression in all the tissues by housekeeping gene, β-actin except muscle tissue (Fig. 2.8). High level expression of epinecidin could be observed in gill tissue; moderate level expression in intestine and feeble expression in skin. No expression of epinecidin could be detected in head kidney, liver and muscle (Fig. 2.9).

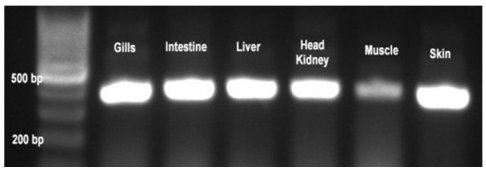


Fig. 2.8: Expression of β actin in various tissues

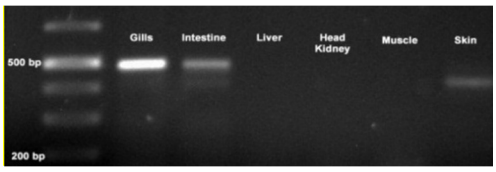


Fig. 2.9: Tissue specific expression of epinecidin with specific primers

2.3.3 Taxonomic Identification using Mitochondrial Cytochrome Oxidae 1 (CO1) gene

There is always ambiguity in the species identification of *Epinephelus coioides, E. malabaricus* and *E tauvina* using morphometric taxonomic

identification system due to similar appearance of premature specimens of these three. To avoid such misperceptions, molecular barcoding was carried out and a 683 bp amplicon was obtained for the mitochondrial CO1 gene with the primers LCO and HCO. Nucleotide blast analysis of the sequence showed 99% similarity with *Epinephelus coioides* and hence the identity was confirmed as *Epinephelus coioides* and the sequence (Fig. 2.10) was submitted to GenBank (ID: KX090374). The genus belongs to Class Teleostei, Sub Class Actinopterygii, Order Perciformes, Family Serranidae.

Cttgattcggtgctgagctggaagtagtaggacagcccttagcctactaattcgagctgagctaag
ccagccgggagctctactaggcgacgaccagatctataatgtaattgttaaagcacatgcttttgta
ataatctttttatagtaataccaattatgattggtggctttggaaactgacttattccacttataatcg
gtgccccagacatagcattccctcgaatgaataatataagcttctgactccttcccccatccttcctgc
ttcttcttgcctcttctggtgtagaagccggtgctggcactggctgaacagtctacccacccctggcc
ggaaacctagcccacgcaggtgcatcagtagacttaactattttctcactacatttagcgggaatttc
atcaattctaggcgcaatcaactttatcacaaccatcattaacatgaaacctcctgctacctctcaat
accaaacacctttatttgtgtgagcagtattgattacagcagtactcctactcctttcccttcccgtcct
tgccgccggcatcacaatgttactcactgatcgtaaccttaataccactttctttgacccagccggag
ggggagacccgattctttaccagcacttattttgattctttggccacaaaaagtctaaa

Fig. 2.10: Nucleotide sequence of CO1 gene amplified from the fish *Epinephelus coioides*.

2.4 Discussion

The 495 bp amplicon obtained from *Epinephelus coioides* collected from Cochin backwaters has shown 99% similarity to the AMP epinecidin-1(AY294407) identified from same species collected from south Chinese Sea,

North West Pacific and 97% similarity to piscidin 2 from *Epinephelus malabaricus* (JX412481). Complete cDNA of epinecidin 1 was 518 bp in length and the aligned portion of epinecidin differ from epinecidin 1 by 10 nucleotides. Likewise, 14 aa difference could be observed between the aligned portion of epinecidin and piscidin 2. However, a 204 bp ORF codes for the 67 aa prepropeptide in all three cases and there was no difference in amino acid sequences in prepropeptide among these peptides. A singe nucleotide difference was there in the ORF of these peptides, but it never reflected in the amino acid sequence of the peptide due to degeneracy of the genetic code. It could be assumed from the findings that rather than mere morphological similarities exhibited by the species, *E. coioides* and *E. malabaricus* might be similar in genetic makeup also. Exquisite similarity between epinecidins identified from the same species collected from geographically remote destinations strengthens the assumption that epinecidin (type 1 piscidin) is highly conserved and remained as a consistent piscidin isoform beyond geographical differences and selection pressures exerted by the surroundings.

Pisicidins and allied peptides identified from various teleosts exhibited considerable differences in amino acid sequence of the mature and propeptide regions which is evident from the ClustalW multiple alignment. Positive Darwinian selection and accelerated rate of amino acid substitution at specific amino acid positions are the reasons for the diversification of piscidin and hepcidin isoforms in fishes (Padhi and Verghese 2007). Three positively selected sites (amino acid positions) have been identified in the mature peptide regions of piscidins identified before and they are at position 25, 29 and 43 (Fernandes *et al.* 2010). Generally

significant variations could be observed at these positively selected sites in the piscidin isoforms of various teleosts. This is due to diversification through mutation and non-synonymous substitution in the selected sites to cope up with the rapidly evolving pathogens (Fernandes *et al.* 2010). However, no such demarcating adaptive selection could be observed at positively selected sites of piscidins identified from *Epinephelus* except for type 3 peptides. Non synonymous substitution of asparagine (N)/histidine (H) and arginine (R) instead of tyrosine (T) could be seen at position 43 of rest of the piscidins. As fishes live in a dynamic ecosystem dominated by opportunistic pathogens, such positive adaptive changes are essential. On the other hand, negative purifying selection is also plausible, since the targets are quite specific (Genus specific pathogens) (Fernandes *et al.* 2010). This could be the reason for high sequence conservation of piscidins identified from the genus *Epinephelus*. The difference in substitution rate is due to variation in selective pressure and the hyper divergence in mature peptide region owing to the elevated mutation rate (Nicolas *et al*. 2003; Vanhoye *et al*. 2003).

Tissues like gills, intestine, skin and muscle were selected for tissue specific expression based on the proximity of continuous contact with pathogens (Silphaduang *et al.* 2006), while head kidney and liver are selected as they are the widely recognized source of various AMPs (Bao *et al.* 2005; Shi and Camus 2006; Dezfuli and Giari 2008; Dezfuli *et al.* 2010). Of the seven tissues tested, epinecidin gene expression could be detected in gills, intestine and skin only. Piscidins are expressed mainly in gills, intestine and skin in teleosts, though ubiquitous expression was also reported from certain fishes like Atlantic cod (Dezfuli *et al.* 2010;

Fernandes *et al.* 2010; Browne *et al.* 2011; Masso-Silva and Diamond 2014). Piscidins are primarily identified from the mast cells of hybrid striped bass, later it has been recognized from phagocytic cells (acidophilic granules), rodlet cells and various other tissues of higher and lower teleosts (Silphaduang and Noga 2001; Silphaduang *et al.* 2006; Mulero *et al.* 2008). Amplification of epinecidin in blood, gills, skin and intestine may be due to the presence of piscidin-positive mast cells that get infiltrated into gills, skin and intestine as they are the primary portals of pathogen entry (Dezfuli *et al.* 2010). Furthermore, evidences suggest that these mast cells circulate within gill microcirculation and kill pathogens either by intracellular (phagocytosis) or extracellular (degranulation of phagosomes) mechanisms. Piscidins are known to assist phagocytosis as they are stored in mast cells and phagocytic granules along with histamines and many other antimicrobial substances (Powell *et al*. 1990; Ellis 2001) whereas extracellular killing is executed by migration to infected gills, intestine and skin and degranulation (Reite and Evensen 2006; Mulero *et al.* 2008). Piscidins are highly potent AMPs that might aggregate on cell surface of pathogens, forming well defined pores together with membrane fragmentation and subsequent leakage of cellular components and cell death (Campagna *et al*. 2007; Olivieri *et al*. 2015). Though no expressed epinecidin was detected in the rest of the tested tissues, this does not mean that these tissues are absent for epinecidin. The absence of expressed mRNAs in these tissues could be the reason for the absence of epinecidin as these sites are not in direct contact with pathogens.

Phylogenetic analysis of amino acid sequences of known teleost pisicidins clearly portrayed its common ancestral origin and pervasive

distribution among lower as well as higher teleosts. Presence of pisicidins and allied peptides in paracanthopterygian fishes like cods, acanthopterygian Antarctic icefish like *Chionodraco homatus* and other perciformes (Moronidae, Sciaenidae, Sparidae, Serranidae, Latidae, Siganidae, Belontidae, Cichlidae, Percichthyidae and Channichthydae) ascertain its wide spread distribution and vital role as evolutionarily conserved effector molecules of primary immune system of teleost (Fernandes *et al.* 2010; Browne *et al.* 2011; Buonocore *et al.* 2012). Nevertheless, piscidins from *Epinephelus* sp. formed a single major cluster in the phylogenetic analysis; they also showed alignment with rest of the piscidin clusters. This supports assumption of negative purification selection by conservation and positive adaptive selection by diversification of piscidins in teleosts. Highest numbers of piscidins are reported from the genus *Epinephelus* and hence it could be used further for future analyses of positive and negative selection pressures in piscidin. However, pleurocidins have been accepted as a member of Piscidin family since they shared similar gene structure and peptide characteristics, they formed separate cluster and structured as an out group in the phylogenetic tree. Though pleurocidins belonged to piscidin family, they are exclusively conserved and positively selected among the order Pleuronectiformes only. This could be the reason for the separation of pleurocidins as an out group. A number of piscidin isoforms had been identified and characterized from the genus *Epinephelus*, yet there was no proper classification or grouping of these peptide rather than mere arbitrary numbering based on order of sequences identified. Results of amino acid sequence analysis as well as phylogenetic analysis clearly indicated four types of peptides from the genus. Of the four piscidin types,

first two are more common and undergone least adaptive diversification, especially at positively modified sites. However, type 3 and 4 are greatly modified in positive selection sites and are very much different in size and amino acid composition when compared to rest of the reported piscidins from the genus.

Despite the differences in nucleotide sequence, epinecidin identified from *E. coioides* showed 100% similarity in amino acid sequence with previously reported epinecidin-1 from the same species of Chinese origin. Irrespective of the geographical differences, high selective pressures and gene duplication events, piscidin/epinecidin of the genus *Epinephelus* retains its amino acid composition and organization as constant without any alterations. Though nucleotide sequence showed slight variation, it is masked and the peptide remains the same without any mutations and variations. Geographical as well as ecological differences do not trigger any positive selection and subsequent modification in epinecidin. Instead these differences preserve the sequence as such, probably by negative purifying selection. Four different class types of piscidins could be differentiated from the genus *Epinephelus*. Of which type 1 and 2 are similar to other reported piscidins. Type 1 and 2 differ from each other in the conserved sequence at N- and C-terminus. 'FIF' sequence at N-terminus and RRRH at C-terminus for the former and a 'FFF' sequence at N-terminus and RRRHRH at the C-terminus of the mature peptide for the later. Type 3 is little more different and is with amino acid sequence FGL at the N-terminus and RRGEEQ at the C-terminus, whereas, type 4 is highly diverse and modified from the rest of piscidins identified from the genus as well as other fishes. Probably, highest number of piscidins could be

identified and characterized from the genus *Epinephelus* and is an indicative of its importance in primary defense of the fish. Moreover, all these observations point out to the fact that piscidins are wide spread among teleosts irrespective of their phylogenetic position and they serve as best examples of positive adaptive evolution and negative purifying selection.

Chapter 3

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF HISTONE DERIVED ANTIMICROBIAL PEPTIDES FROM MARINE FISHES

Contents

3.1 Introduction

Histones are primarily DNA-binding proteins acting as spools around which DNA winds up to form condensed chromatin organization particular to eukaryotic cell nucleus. Nucleosome is the basic structural unit of chromatin organization and is fabricated of nucleosome core particle formed by two copies of each of core histones (H2A, H2B, H3 and H4) which are linked together by the linker histone, H1 (Tagai *et al.* 2011a). Besides the core histones and linker histones that are integral parts of chromatin organization, various cytoplasmic histone variants and un-acetylated histones also have been reported recently (Cho *et al.* 2002a,b; Talbert and Henikoff 2014). These histones are used as weapons in conflicts between parasites and their hosts and are also responsible for responses to environmental perturbations, chromatin repair etc. (Talbert and Henikoff 2014). Recent scientific interventions recognize histones as

multifunctional proteins involved in multitude of biological functions beyond the confines of the nuclear envelope (Parseghian and Luhrs 2006). For instance, histone has pivotal role in DNA replication, transcription control, homeostasis (Reichhart *et al.* 1985), endotoxin-neutralization (Kim *et al.* 2002), leucocytes stimulation (Pedersen *et al.* 2003), apoptotic signal transmission (Konishi *et al.* 2003) and host defense i.e., acting as proteins/peptides with antimicrobial activity (Thatcher and Gorovsky 1994; Talbert and Henikoff 2014). First report of antimicrobial property of histone was in 1942 (Miller *et al.* 1942), but the concept remained unattended till the mid-1990s, until the report of histone proteins as antimicrobial agents in wound blister fluids got published in 1996 (Frohm *et al.* 1996).

First report on the antimicrobial property of histones in fishes was from the skin mucus extracts of the wounded catfish, *Parasilurus asotus* as a 19 peptide named as parasin I (Park *et al.* 1998b). Out of the 19 amino acids of parasin I, 18 amino acids were identical to the N-terminus of buforin 1, a 39 aa peptide derived from the N-terminus of toad histone H2A (Kim *et al.* 1996). Parasin I has shown promising antimicrobial property against wide spectrum of microorganisms without any haemolytic activity (Park *et al.* 1998b). Since then reports began to emerge out and the number of intact or fragmented histones functioning as host defense armaments is surprisingly high in both vertebrates and invertebrates (Talbert and Henikoff 2014; Chaurasia *et al.* 2015). Histones are highly alkaline peptides rich in lysine/arginine residues, which make them effective and selective antimicrobial agents against negatively-charged target cell membranes. Among the five histone types, histone H1, H2A and

H2B are rich in lysine residues while H3 and H4 are rich in arginine residues (Tagai *et al.* 2011a). Antimicrobial property has been reported from all the five types of histone proteins. Besides, surprising sequence similarity of several naturally occurring AMPs with certain regions of various histone subtypes approves the role of histones in host defense (Kawasaki and Iwamuro 2008).

Though histone-derived antimicrobial peptides (HDAPs) have been reported from all five classes of histone proteins, histone H2A derived AMPs lead the chart in terms of number. Parasin I (Park *et al.* 1998b), hipposin (Birkemo *et al.* 2003), buforin I and II (Cho *et al.* 2009b), abhisin (Zoysa *et al.* 2009), himanturin (Sathyan *et al.* 2012a), sunettin (Sathyan *et al.* 2012c) molluskin (Sathyan *et al.* 2012b), harriottin (Sathyan *et al.* 2013) and sphistin (Chen *et al.* 2015b) represent histone H2A derived AMPs. Besides, H2A like HDAPs have also been identified from the tissues of Pacific white shrimp, *Litopenaeus vannamei* (Patat *et al.* 2004) and Giant freshwater prawn *Macrobrachium rosenbergii* (Arockiaraj *et al.* 2013). Likewise H2B derived HDAPs have also been identified from the skin mucus secretions of Channel catfish, *Ictalurus punctatus* (Robinette *et al.* 1998), Schlegel's green tree frog *Rhacophorus schlegeli* (Kawasaki *et al.* 2003) and acidified tissue extracts of American oyster *Crassostrea virginica* (Seo *et al.* 2005). HDAPs also could be identified from the lysine-rich linker histone, H1. Strong antimicrobial agents characterized from acidified tissue extracts of Salmon, *Salmo salar* (Richards *et al.* 2001), Rainbow trout, *Onchorynchus mykiss* (Fernandes *et al.* 2002) and Olive flounder, *Paralichthys olivaceous* (Nam *et al.* 2012) belonged to this group. HDAPs derived from arginine-rich histones that is H3 and H4 are

relatively less abundant in number but have been reported from *L. vannamei* (Patat *et al.* 2004) and *M. rosenbergii* (Tagai *et al.* 2011a; Chaurasia *et al.* 2015).

Lysine-rich histone derived AMPs are astonishingly common in skin mucus secretions of fishes and amphibians. Presence of HDAPs in primary defense barriers like skin mucus as principal humoral effector component accentuates its significance as an AMP. In addition, induced expression of HDAPs could be observed in various tissues of fish (liver, intestine, stomach and testes) (Richards *et al.* 2001) and haemocytes of white shrimp and Scallop, *Clamys ferrari* (Patat *et al.* 2004; Li *et al.* 2007). However, the significance of intact or fragmented histones in host defense of lower vertebrates is well known, the actual mechanisms by which histones, that are integral part of chromosomes residing inside nucleus traverse to body secretions was not known until the production of buforin 1 was recorded. Buforin 1 was produced as a result of enzymatic cleavage of cytoplasmic non-acetylated histone H2A that is secreted into the gastric lumen of Korean frog (*Bufo bufo gargarizans*) by endogenous protease pepsin at position 39 (Kim *et al.* 2000). Later, wound-induced production of a 19 aa HDAP, parasin I was also discovered. Parasin I produced is inducibly upon injury against wound inflicted secondary infections by the action of matrix assisted metalloprotease activated cathepsin D. This cathepsin D will in turn cleaves the N-terminus of histone H2A of catfish at position 19 which results in the production of highly active broad spectrum AMP parasin I (Cho *et al.* 2002a,b). Detailed investigation on histone precursors exposed the existence of two populations of histones, acetylated H2A and un-acetylated H2A. The

un-acetylated histone H2A that remains in the cytoplasm after migration to nucleus gets accumulated eventually in the cytoplasm and serves as precursors for HDAPs (Cho *et al.* 2002b).

Recently another mechanism of expulsion of histone proteins/ chromatin *via* reactive oxygen species (ROS) by neutrophils and other phagocytic cells as neutrophil extracellular traps (NETs) has been reported (Palić *et al.* 2007). The discharged chromatin form complex meshes decorated with histones (H1, H2A, H2B, H3 and H4), enzymes (cathepsin D, elastase, neutrophil elastase, NADPH oxidases, proteinase 3 etc.) and AMPs (cathelicidins, defensins and LL37) that entrap and kill bacteria, fungi, viruses and other parasites (Brinkmann *et al.* 2004; Guimarães-Costa *et al.* 2012; Robb *et al.* 2014). The mechanism explicates another possible chance of occurrence of cytoplasmic histone proteins that may serve as potential precursors for HDAPs.

Electrostatic attraction of AMPs to negatively charged microbial membranes, resulting in destabilization and pore formation of planar lipid bilayer resulting in leakage of cytoplasmic content leading to the death of the organism is the most common and unanimously accepted mode of action of AMPs. Though the exact mechanisms of action of HDAPs are not well studied, recent reports suggest that most of the HDAPs could electrostatically bind to anionic lipid membrane owing to their highly cationic nature (Strömstedt *et al.* 2010). Most of the HDAPs kill target cells by membrane disruption and subsequent lysis of the cell as in the case of histone H2A isolated from trout mucus (Fernandes *et al.* 2002). They did not form stable ion channels, instead will create an overall perturbation in

membranes and the mode of action could classify basically as 'carpet model'. Parasin I, a well-studied HDAP exert antimicrobial activity through pore formation but, unlike micellization as in the case of hipposin and other histone H2A derived peptides, parasin forms barrel staves lined with α-helical peptides leading to pore formation and cell lysis (Zhao *et al.* 2015). An almost similar mechanism is exhibited by buforin II also. Alpha helical conformation of the peptide has pivotal role in this type of mircobicidal mechanism. Buforin II (Xie *et al.* 2011), one of the most studied AMPs and bacterial outer membrane protease cleaved histone H2B-derived HDAP exhibits another mechanism of action (Kawasaki *et al.* 2008). They do not lyse the cell, instead penetrate into the cell and find out cytoplasmic targets or activates intracellular cascades leading to the death of the organism (Lee *et al.* 2008). Histones H3 and H4 remained on the cell surface and subsequently disrupted the cell membrane structure with bleb formation in a manner similar to general antimicrobial peptides (Pavia *et al.* 2012).

This chapter deals with molecular and functional characterization of histone-derived AMPs identified from the Bengal tongue sole *Cynoglossus semifasciatus* and catfish *Tachysurus jella* (*Arius jella*). The chapter also extends to delineate evolutionary significance of histone proteins, especially H2A.

3.2 Materials and methods

3.2.1 Sample collection

Live catfish *T. jella* (Super Class Pisces, Class – Osteichthys, Subclass Actinopterygii, Order-Perciformes, Family-Tachysuridae, Genus-*Tachysurus*) and Bengal tongue sole, *C. semifasciatus* (Super Class Pisces, Class–

Osteichthys, Subclass-Actinopterygii, Order-Pleuronectiformes, Family-Soleidae, Genus-*Cynoglossus*) were collected from Kalamukku harbour, Kochi, Kerala, India. Samples were transported to laboratory in live condition.

3.2.2 RNA extraction

Blood was collected from the lamellar artery near gill region of the fishes (*T. jella* and *C. semifaciatus*) using 5 ml syringes (RNase free) rinsed in precooled anticoagulant solution (RNase free 10% sodium citrate, pH 7). Total RNA was extracted from the blood cells of the fishes with TRI® Reagent in accordance with the manufacturer's instructions. For details on RNA extraction procedure refer section 2.2.4 of Chapter 2.

3.2.3 cDNA synthesis

The first strand cDNA was synthesised by reverse transcription in a 20 µl reaction mixture containing 5 µg total RNA. For details on cDNA synthesis refer section 2.2.6 of Chapter 2.

3.2.4 PCR amplification

The PCR amplification of a hipposin-like gene from the cDNA of the fishes was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer, and 1U Taq DNA polymerase using the forward primer (5'-ATGTCCGGRMGMGGSAARAC-3') and reverse primer (5'-GGGATGATGCGMGTCTTCTTGTT-3'). The PCR conditions involved an initial denaturation of 94 °C for 2 min followed by 35 cycles

of 94 °C for 15 s 60 °C for 30 s, and 68 °C for 30 s and a final extension at 68 °C for 10 min.

3.2.5 Agarose gel electrophoresis

The PCR product (10 µl) was analyzed on 1.5% agarose gel. See section 2.2.8 of Chapter 2 for details on agarose gel electrophoresis.

3.2.6 Sequencing

Sequencing of the purified PCR products were done with ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 377 DNA sequencer (Applied Biosystem) at SciGenom Sequencing Facility, Kakkanad, Kochi, India.

3.2.7 Sequence analysis and molecular characterization

The sequences were analysed, trimmed and assembled using GeneTool software. The cDNA-based gene sequences were translated using Expert Protein Analysis System (ExPASy). Homology searches of nucleotide sequence and the deduced amino acid sequence were performed using BLASTn and BLASTp algorithm of the NCBI. Peptide characterization and further analysis of the amino acid sequence was performed using various computer based algorithms as explained in section 2.2.10 of Chapter 2.

3.2.8 Phylogenetic analysis

Previously reported histone H2A sequences of both vertebrate and invertebrate organisms were retrieved from GenBank and multi-aligned using ClustalW and GeneDoc computer programmes. Phylogenetic tree

was constructed by Neighbor Joining method using the software MEGA 5.05. For details refer section 2.2.11 of Chapter 2.

3.3 Results

3.3.1 Sequence analysis

A 245 bp amplicon coding for 81 aa (Fig. 3.1) was amplified from the blood cells of Bengal tongue sole *C. semifasciatus* (Fig. 3.2) and catfish *T. jella* (Fig. 3.3). BLAST analysis of the nucleotide and deduced amino acid sequence revealed the identity of both the sequences as histone H2A. The nucleotide and deduced amino acid sequence of the amplicon are shown in Fig. 3.4. Though the nucleotide sequences of the fishes exhibited considerable variation among themselves, the deduced amino acid sequences were found to be almost similar with a single exception at amino acid position number 78, where Arg^{78} in *C. semifasciatus* is replaced by Ser^{78} in *T. jella*. A high degree of degeneracy of genetic code could be observed among the sequences. Both the nucleotide and deduced amino acid sequence of the peptides obtained from the fishes, *C. semifasciatus* and *T. jella* have been submitted to GenBank (ID: HQ720152 and HQ720153, resp.). ClustalW multiple alignment (Fig. 3.5) of the peptide sequences with previously reported HDAPs revealed that the first 51 amino acids from the N-terminus (excluding the initiator methionine) of the peptide were similar to the AMP hipposin reported from the skin-mucus of Atlantic halibut *Hippoglossus hippoglossus*. The only difference noted was His^{41} in hipposin, which is replaced by Glu^{42} in both the peptides reported from the marine bony fishes *C. semifasciatus* and *T. jella*.

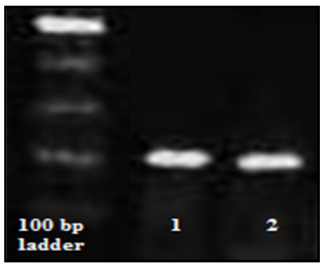


Fig. 3.1: 245 bp amplicon of teleostin from *Cynoglossus semifasciatus* and *Tachysurus jella*

Fig. 3.2: *Cynoglossus semifasciatus*

Fig. 3.3: *Tachysurus jella*

(a)

```
                  *         20        *         40        *         60        *         80        *        100        *
Tachysurus : ATATGTCCGGGCGCGGCAAGACGGGAGGAAAGCCGTGCTAAGGCAAGACGCGCAGCTCCGCGCTGGTCTACAATTACGTGTGGGCCGCGTGCAACGTCTTGCGCGAAGGGA : 117
Cynoglossu : TAATGTCCGGGCGCGGCAAGACAGGCGGTAAGGCCAGACGGCTAAGGCCAAGACGCGTTCTTCCAGCGCTGGACTCCGTTCCCGTGGGCCCTGTTCATAGCTGTCAGGAAGGGTA : 117
             T ATGTCCGGGCGCGGCAAGAC GG GG AA GCC G GCTAAGGC AAGAC CG    TCC G GCTGG CT CA TT CC GTGGGCCG GT CAT G CT CT  GGAA GG A

                  120       *       140       *       160       *       180       *       200       *       220       *
Tachysurus : ACTACGCCGAGCGCGTCGCCGCCGGGCTCCTGTGTACTGGCCGCTGTCGCTCGAGTATCTGACCGCTGAGATCCTGGAGTTGGCGGTAACGCTGCCCGGGACAACAAGAAAACCT : 234
Cynoglossu : ACTACGCCGAGCGCGTTGCTGCCGCTGCTCCTGTGTATCTGGCGGCGTGCTCGAGTATCTGACCGCTGAATCTTGGAGTTGGCAGAACGCTGCCCGTGACAACAAGAAGACCC : 234
             ACTACGCCGAGCG GT GG GCCGG GCTCCTGTGTA CTGGC GC GTGCTCGAGTATCTGACCGCTGA ATC TGGAGTTGGC GG AACGCTGCCCG GACAACAAGAA ACC

                  240
Tachysurus : GCATCATCCGT : 245
Cynoglossu : GCATCATCCGA : 245
             CATCATCCC
```

(b)

```
                  *         20        *         40        *         60        *         80
Cynoglossu : MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTRIIP : 81
Tachysurus : MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKTSIIP : 81
             MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLLRKGNYAERVGAGAPVYLAAVLEYLTAEILELAGNAARDNKKT IIP
```

Fig. 3.4: Nucleotide (a) and deduced amino acid (b) sequences of histone H2A from *C. semifasciatus and T. jella*

Fig. 3.5: ClustalW multiple alignment of teleostin with previously reported histone H2A derived AMPs, from fishes, molluscs and crustaceans.

3.3.2 Molecular characterization of the peptide

Peptide cutter tool of ExPASy was employed to identify specific enzyme cleavage sites resulting in the formation of 52 aa peptide similar to hipposin and the algorithm predicted that the proteolytic enzymes chymotrypsin, pepsin, proteinase K, and thermolysin have cleaving sites at the position 52 of the peptide suggesting the possibility of formation of hipposin-like fragment from the N-terminus of the peptide. Considering the exact similarity of amino acids between the peptides and high degree of conservation of the particular region among other teleost fishes this 52 aa peptide corresponding to hipposin was termed 'teleostin' and hereafter will be referred by this term.

Peptide characterization using ProtParam tool revealed that the 52 aa teleostin would have a predicted molecular weight of 5.527 kDa, a theoretical isoelectric point of 12.01, and a net positive charge of +12 contributed by 8 arginine and 5 lysine residues against a single glutamine residue. The peptide was found to exhibit a total hydrophobic index of 32% contributed by amino acids Ala (13%), Val (7%), Leu (7%), Met (1%), and Phe (1%). The amino

acids glycine and arginine have a higher percentage contribution to the total amino acids of the peptide, 17% and 15%, respectively. Protean programme of DNASTAR Lasergene 10 Core Suite predicts teleostin to have a concentration of 1.86 mg/ml for an absorbance of 1 OD at 280 nm, and 1 µg of the peptide would contain 180.52 pMole. Antimicrobial peptide database predicts teleostin to be an AMP with a protein-binding potential of 2.38 kcal/mol.

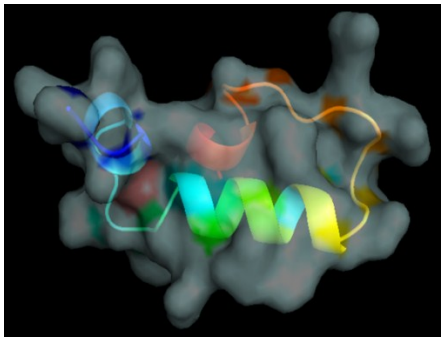


Fig. 3.6: Predicted three dimensional structure of teleostin, created using the software PyMol.

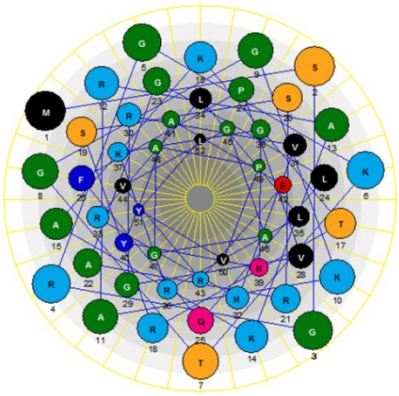


Fig. 3.7: Schiffer-Edmundson helical wheel diagram of teleostin created with DNAstar program demonstrating the possible amphipathic α-helical conformation. Helical wheel projects the arrangement of amino acids and residue numbers are counted from the amino (N) terminus of teleostin.

SWISS-MODEL work space was used to predict the three dimensional structure of teleostin and the software predicted the structure as an α-helix with two extended loops on either sides of the helix (Fig. 3.6). The main helix was formed of 9 aa starting from Val^{28} to Arg^{36} of the peptides. Like-wise two extended loops on either side of the helix were formed of amino acids Gly^{23} to Pro^{27} and Lys^{37} to Ala^{46}. Two small helices also could be observed at both termini and are formed of the amino acids from Arg^{18} to Ala^{21} and Lys^{47} to Leu^{42}. Schiffer- Edmundson helical wheel modelling further confirmed the helical structure of the peptide as the hydrophobic and hydrophilic residues arranged on opposite sides of the wheel (Fig. 3.7).

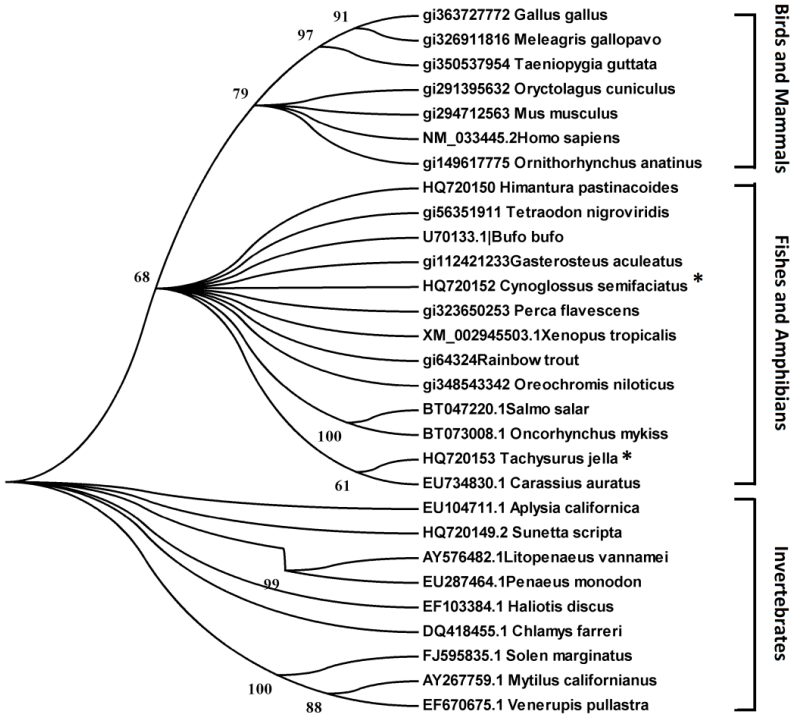


Fig. 3.8: A bootstrapped neighbor-joining tree obtained using MEGA version 5.05 illustrating relationships between the nucleotide sequence of *C. semifasciatus* and *T. jella* to the nucleotide sequences of previously reported histone-H2A from various vertebrate and invertebrate organisms.

3.3 Phylogenetic analysis

A boot-strapped phylogenetic tree was constructed based on the nucleotide sequence of histone H2A reported from invertebrates and vertebrates to delineate the evolutionary significance and conservation of histone sequences among themselves. The phylogenetic tree thus formed could be easily differentiated into three main clusters, bottom cluster representing invertebrates, middle one representing fishes and amphibians and the third one representing birds and mammals (Fig. 3.8). Teleostin found aligned with the clade of fishes and amphibians indicate its close resemblance with other teleost fishes.

3.4 Discussion

Histone proteins are rich in cationic amino acids, arginine and/or lysine and hence have a net positive charge, which is the prime requisite for an AMP (Boman 2003; Tagai *et al.* 2011a). Histone H2A is one among the core DNA-binding histones that is rich in lysine residues (Tagai *et al.* 2011a). The N-terminus region of histone H2A is classically associated with DNA binding and is the region of nuclear signal localization (Balicki *et al.* 2002). Concurrently, it is also reported to serve as a precursor for various AMPs (Park *et al.* 1998a; Birkemo *et al.* 2003; Cho *et al.* 2009b). Blast analysis of the nucleotide and deduced amino acid sequence confirmed the identity of the peptide as histone H2A. The N-terminus of the peptide showed striking similarity with 51 aa HDAP hipposin identified from Atlantic halibut, *H. hippoglossus* except for the initiator methionine and the replacement of the basic amino acid histidine by an acidic amino acid glutamine. Though the nucleotide sequence obtained from the fishes

exhibited remarkable degree of variations, the amino acid sequence remains the same. Such conservation of sequences could be observed throughout the members of the super order Teleostei due to lower rate of evolution of histone H2A genes (Thatcher and Gorovsky 1994). Owing to the similarity with teleost hipposin and other reported teleost histone H2A proteins, the peptide was selectively named as teleostin after the super order Teleosteii.

Fragmented histone proteins like parasin, buforin, hipposin, onchorhyncin etc. have been detected from the skin mucus secretions of fishes and amphibians. Exact mechanism by which all these peptides are formed is not yet known. However, the production of parasin by cathepsin D and buforin 1 by endopeptidase pepsin from the N terminus of histone H2A have been hypothesized and proved (Kim *et al.* 1996; Cho *et al*. 2002b), the mechanism by which hipposin/teleostin is cleaved off from the histone precursor has never been reported. Hence peptide cutter tool was employed to analyze the potential enzymes and possible cleavage sites that may result in the formation of teleostin. The tool predicted that the proteolytic enzymes chymotrypsin, pepsin, proteinase K and thermolysin have cleaving sites at various positions of the N-terminus of the peptide. The proteolytic activity of these enzymes on the protein in question would release a fragment similar to teleostin. Bacterial outer membrane proteases are also reported to assist in induced production of HDAPs from precursor histones (Tagai *et al.* 2011b). Like-wise, the possibility of infection-induced production of teleostin by bacterial outer membrane proteases from histone H2A precursor also could not be excluded. Production of HDAP parasin I from *Parasilurs asotus* by matrix metalloprotease

activated cathepsin D upon the epidermal injury of the host as primary defense against injury and imminent invasion of microbes is a classic example of induced production of HDAPs (Cho *et al.* 2002a,b). Either cytoplasmic excess un-acetylated histone H2A or those histones which are expelled to the exterior by phagocytic neutrophils/monocytes through extracellular traps could serves as precursors for the production of HDAPs including teleostin (Cho *et al.* 2002b; Palić *et al.* 2007; Kaplan and Radic 2012; Robb *et al.* 2014). In the former case, differentiation of histone precursors to HDAPs is stimulated by injury/infection, while in the latter chromatin is released only when there is an infection or inflammation. Bacterial LPS and β-Glucan induced expulsion of chromatin by kidney neutrophils of fish has been reported (Palić *et al.* 2007).

The 52 aa teleostin representing the N-terminal region of histone H2A protein has been characterized to be a cationic, amphipathic molecule that can configure to an α-helical conformation (Birkemo *et al.* 2003). Hipposin is an AMP devoid of any acidic amino acids and hold a net charge of +13. Teleostin was found to have a net charge of +12. The reduced cationicity of teleostin is mainly because of replacement of basic aa His^{41} in hipposin by acidic aa Glu^{42} in teleostin, which would reduce the cytotoxic activity of the peptide and make it more specific to acidic bacterial membranes (Khandelia and Kaznessis 2006; Khandelia *et al.* 2008). Hipposin is among the most potent antimicrobial peptides discovered to date (Birkemo *et al.* 2003, 2004). It exhibited broad spectrum activity against several Gram-positive and Gram-negative bacteria and the activity could be detected down to a concentration of 1.6 µg/ml (Birkemo *et al.* 2003). Similar anti-microbial properties could also be expected for

teleostin, since both teleostin and hipposin have similar amino acid composition, structural conformation, helical property and amphipathicity. The replacement of His^{41} of hipposin by Glu^{42} in teleostin has not made any alteration in helical property as well as amphipathicity of the peptide, hence it could be concluded that teleostin would show an activity profile comparable to hipposin.

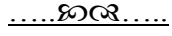
The mechanism of action of AMPs is always a matter of controversy, yet membrane disruption-mediated osmotic imbalance and subsequent bio-energetic collapse of the cell is the most convincing mode of action (Yount *et al.* 2006). Positively charged teleostin can easily interact with negatively charged target cell membranes and can bring about the disruption of membrane equilibrium by creating stable ion channels as in buforin I (Cho *et al.* 2009b) or parasin I (Koo *et al.* 2008) or by direct disintegration and micellization of target cell membrane as in case of histone H2A isolated from trout mucus (Fernandes *et al.* 2002). Very recently, the mode of action of hipposin was discovered and it was the destruction of target cell membranes by destabilization and micellization leading to transient pore formation. Unlike buforin and parasin I, hipposin could never form stable ion channels (Bustillo *et al.* 2014). It also lacks cell penetrating ability as that of buforin II. Helical property of the peptide has significant role in determining the antimicrobial activity (Koo *et al.* 2008). Teleostin is an α-helical peptide similar to hipposin in all aspects defining biological activity and hence it could interact with the hydrophobic phospholipid heads of the membrane and bring about the lysis of bacterial cells by cell membrane disruption in a similar way to that of hipposin.

The enzyme cathepsin D produces parasin I in *P. asotus* (Cho *et al.* 2002b). Similarly pepsin produces buforin I in *Bufo bufo gargarizans* and buforin II from buforin I by endoproteinase lys-C cleavage (Cho *et al.* 2009b). Specific proteolytic cleavage of 52 aa teleostin would give rise to three more peptides, a 19 aa parasin-like fragment, a 39 aa buforin I-like fragment and 21 aa residue buforin II-like fragment corresponding to 2-20 aa, 2-40 aa and 17-37 aa of the teleostin respectively. The 19 aa parasin-like and 39 residue buforin I-like analogues of teleostin differ from that of parasin and buforin I in one and three amino acids respectively, while the portion of teleostin analogous to buforin II is exactly the same. The peptides that would be formed from teleostin by specific enzymatic cleavage could function separately in a mutually independent manner with differential modes of action. Recent investigations reveal that the N-terminal parasin I-like peptide is responsible for the membrane lytic potential of hipposin as well as teleostin. Peptide lacking N-terminal sequence would function like buforin II. The C-terminal peptide is biologically inactive but could penetrate into the target cells where it exerts no significant cellular modulations to arrest or kill the invader. Hence the C-terminal peptide could be used effectively as a cell penetrating peptide in drug delivery system and related application (Bustillo *et al.* 2014).

The NJ phylogenetic tree based on nucleic acid sequences of histone H2A reported from wide range of organisms indicate a common ancestral origin of invertebrate and vertebrate histone proteins and their adaptive divergence to present positions. The tree branched into three main clusters. The trend of evolution could be easily calculated from the clustering pattern it-self. Invertebrates formed a separate lineage and cluster which is

distant from whole vertebrates. On the vertebrate main branch, two clusters could be seen. One consisted of fishes and amphibians and the other one by birds and mammals. Clustering of fishes cum amphibians and birds cum mammals together indicates evolutionary pathway of fishes to amphibian to higher mammals. Reptilian representatives are missing yet route of evolution is very clear from the cladogram. Histone H2A sequence of *C. semifasciatus* and *T. jella* aligned along with the fishes in the cluster formed by fishes and frogs in accordance with its sequence similarity. Significant overlap was observed in the histone H2A nucleotide sequences of 'amphibians and fishes' and 'birds and mammals' from which it could be inferred that histone proteins remain relatively unchanged throughout the course of evolution.

Histone proteins and their derivative peptides play inevitable role in the immune defense of many organisms especially in fishes, as they rely more on the rapid, non-specific innate immune parameters than short lived adaptive immune responses. Accumulating evidences on wide spread presence of fragmented and intact histones in the skin mucus secretion and essential tissue extracts also emphasize the same. The 52 residue α-helical cationic peptide teleostin identified from teleost fishes possess all characteristic features of classical AMPs, endorsing it to be an antimicrobial peptide with potential to develop into novel therapeutic agents. Moreover it could be used as templates for the development of hybrid/stabilized AMPs and for the preparation of peptide cocktails.



Chapter 4

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF TWO HEPCIDIN ISOFORMS FROM MARINE FISHES

Contents

4.1 Introduction

Hepcidin is an acute phase short cysteine-rich peptide of multiple physiological functions. It was identified primarily as Liver Expressed Antimicrobial Peptide (LEAP-1) from the human blood ultra-filtrate (Krause *et al*. 2000). Later it was identified from human urine and named as hepatic antimicrobial peptide (HAMP), also referred to as hepcidin synonymously because of its hepatic origin and antimicrobial property (Park *et al.* 2001; Singh *et al.* 2011). Hepcidins are generally synthesized as a preprohepcidin (≈ 80-90 aa) comprising of a signal peptide (24-26 aa), a variable pro region (≈ 40 aa) and a mature bioactive peptide (20-31 aa) containing 4-8 cysteine residues which will form the disulphide bond backbone of the β-hairpin. Hepcidin-like peptides are widely distributed among vertebrates with representatives from all classes including fishes.

Hepcidin gene consists of three exons and two introns. In all organisms from which hepcidins are reported, each exon corresponds to three domains of the hepcidin precursor. In fishes, though the expression of hepcidin genes could be detected in all tissues, liver is presumed to be the major site of hepcidin synthesis (Solstad *et al.* 2008). However, hepcidins are synthesized as inactive precursor in fishes, cleavage of leader signal peptide and subsequent propeptide from the precursor by signal peptidases and propeptide convertase respectively will result in the formation of mature bioactive peptide. Primarily the signal peptide will be cleaved off and the resultant prohepcidin will be exported to the lumen of endoplasmic reticulum where it undergoes a second cleavage after the conserved motif RXK/RR to obtain the mature bioactive peptide, probably by furin and related propeptide convertases like PC5, PC7 and PACE4 (Schranz *et al.* 2009; Singh *et al.* 2011). The function of propeptide region is to assist the transit of AMPs through the subcellular compartments and to protect the cellular components of host against toxic effects of mature peptide (Valore and Ganz 2008).

Solution NMR structure defined hepcidin as a β-hairpin like structure cross linked with disulphide bonds. The β- hairpin consists of two slightly twisted antiparallel β-sheets connected by a more or less flexible hairpin loop. Despite the sequence variation, almost all known vertebrate mature hepcidins have 8 conserved cysteines. These cysteines will form position specific intramolecular disulphide bonds, three across the stem of the hairpin structure (C1-C8, C2-C7, C3-C6) and one unusual vicinal disulphide bond (C_4 and C_5) near the loop of mature peptide (Hunter *et al.* 2002; Lauth *et al.* 2005; Xu *et al.* 2008). This specific core domain

signature discriminates hepcidin from the rest of the cysteine rich AMPs and the vicinal disulphide bond is presumed to be the key factor regulating antimicrobial property of the peptide (Singh *et al.* 2011; Hocquellet *et al.* 2012) . Besides isoforms with 6-8 cysteine residues, a novel isoform with 4 cysteines was also identified from Antarctic Notothenoid fishes (Xu *et al.* 2008; Wang *et al.* 2009). Positive Darwinian selection and adaptive evolution is considered as the regulating factor for accelerated amino acid substitution and sequence diversity of the gene encoded AMPs. Evidences approve such kind of adaptive evolution and positive Darwinian selection in hepcidins, especially of Perciforms and Pleuronectiform fishes (Padhi and Verghese 2007).

Though the first report of hepcidin was from mammals, adaptive evolution of hepcidin gene is prominent in fishes, since mammals except mice possess a single copy of HAMP gene and fishes possesses almost up to 8 copies of hepcidin genes (Rodrigues *et al.* 2006; Yang *et al.* 2007; Masso-Silva and Diamond 2014; Chou *et al.* 2016). Based on consensus and diversity, HAMPs identified from fishes could be broadly classified into two main lineages *i.e.*, HAMP1 and HAMP2. HAMP1-like peptides are distributed in all groups of fishes and is an orthologue of mammalian HAMP while HAMP2-like peptides reported only from acanthopterygian fishes till date (Hilton and Lambert 2008). These two groups of peptides differ mainly in the overall charge and the presence or absence of a presumed iron binding motif QSHLS/DTHFP. HAMP1-like peptide is characterized by the presence of iron-binding motif at the N-terminus of the mature peptide and in having an overall positive charge for preprohepcidin, prohepcidin as well as mature hepcidin. Conversely, the

iron-binding motif is absent for HAMP2-like peptide and only mature peptide is cationic (Hilton and Lambert 2008). HAMP1-like peptide is iron regulatory as well as antimicrobial in function. Up regulation of HAMP1-like genes after bacterial challenge indicates its dual role as AMP and iron regulator hormone (Rodrigues *et al.* 2006; Yang *et al.* 2013). HAMP2-like peptides are highly diverse in amino acid sequence and are predominantly antimicrobial in nature as it is relatively insensitive to iron overload (Pereiro *et al.* 2012). Hepcidin, assumed to be an iron regulatory hormone as well as an antimicrobial peptide, probably evolved from an antimicrobial peptide gene through mass gene duplication events happened millions of years back (Shi and Camus 2006).

Hepcidin-like peptides have been identified from more than 37 species of fishes. Though the peptide is generally cationic in nature, the existence of anionic hepcidin could not be excluded (Zhou *et al.* 2011). Bass hepcidin was the first non-mammalian hepcidin identified and characterized (Shike *et al.* 2002). Since then, a number of hepcidin isoforms have been identified from both acanthopterygian and non-acanthopterygian fishes. Only hepcidins belonging to HAMP1 group have been identified so far from non-acanthopterygian fishes. This include hepcidins identified from Atlantic cod, *Gadus morhua* (Solstad *et al.* 2008), Salmon, *Salmo salar* (Douglas *et al.* 2003a), Ayu, *Plecoglossus altivelis* (Chen *et al.* 2010), Channel catfish, *Ictalurus punctatus* (Hu *et al.* 2007), Common carp, *Cyprinuc carpio* (Li *et al.* 2013), Japanese rice fish, *Oryzias latipes* (Douglas *et al.* 2003a), Medaka, *O. melastigma* (Cai *et al.* 2012), Mud loach, *Misgurnus mizolepis* (Nam *et al.* 2011) and Snow trout, *Schizothorax richardzoni* (Chaturvedi *et al.* 2014). Hepcidin isoforms

HAMP1 or 2 have been reported from fishes like Barramundi, *Lates calcarifer* (Barnes *et al.* 2011), Black porgy, *Acanthopagrus schlegelli* (Yang *et al.* 2011, 2007), Black rockfish, *Seabastes schlegelli* (Dezfuli *et al.* 2010), Blotched snakehead, *Channa macculatus* (Gong *et al.* 2014), Blunt snout bream, *Megalobrama amblycephala* (Liang *et al.* 2013), Europen Sea bass, *Dicentrarchus labrax* (Neves *et al.* 2011), Gilth head sea bream, *Sparus aurata* (Cuesta *et al.* 2008), Half smooth tongue sole, *Cynoglossus semilaevis* (Wang *et al.* 2012c), Hybrid striped bass (Lauth *et al.* 2005), Olive flounder, *Paralychthys olivaceous* (Douglas *et al.* 2003b; Shi and Camus, 2006), Japanese sea bass, *Lateolbrax japonicas* (Ren *et al.* 2006), Large-mouth bass, *Micropterus salmoides* (Robertson *et al.* 2009), Large yellow-croacker, *Pseudosciana diacanthus* (Wang *et al.* 2009; Zhang *et al.* 2009), Orange spotted grouper, *Epinephelus coioides* (Zhou *et al.* 2011; Qu *et al.* 2013), Red spotted grouper, *Epinephelus akara* (Mao *et al.* 2013), Pacific mutton hamlet, *Alphestes immaculates* (Masso-Silva *et al.* 2011), Red banded seabream, *Pagrus Auriga* (Martin-Antonio *et al.* 2009), Red seabream *Chrysophrys major* (Chen *et al.* 2005; Hsu *et al.* 2011a), Rock bream, *Oplegnathus faciatus* (Cho *et al.* 2009a), Small mouth bass, *Micropterus dolomieu* (Robertson *et al.* 2009), Tilapia, *Oreochromis mossambicus* (Huang *et al.* 2007), Turbot *Scophthalmus maximus* (Pereiro *et al.* 2012), Winter flounder, *Pleuronectus americanus* (Douglas *et al.* 2003b), Zebra fish, *Danio reirio* (Lin *et al.* 2014; Shike *et al.* 2004) and Antarctic notothenoid fishes (Xu *et al.* 2008).

Hepcidin exhibit broad spectrum antimicrobial activity against various Gram-positive and Gram-negative bacteria, fungi and viruses at μM level. Besides, hepcidin also possess immunomodulatory and anticancer properties

(Chen *et al.* 2009a; Wang *et al.* 2010; Pan *et al.* 2011b). Hepcidin gene expression in vertebrates is regulated positively by proteins responsible for hereditary hemochromatosis and bone morphogenetic proteins (BMPs) through SMAD (Sma and Mad Related Family) signalling pathway. There are also reports of an additional regulatory mechanism in fishes which is induced by inflammatory cytokines IL-6 through JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) (Muckenthaler, 2008; Neves *et al.* 2011). Induced expression of hepcidin on bacterial challenge as well as variable expression of hepcidin in different life stages of fish, including larval forms, underscores its evolutionary significance and importance in the innate immune system of fish (Shike *et al.* 2002; Lauth *et al.* 2005; Cuesta *et al.* 2008; Wang *et al.* 2009, 2012c; Masso-Silva *et al.* 2011; Nam *et al.* 2011; Gong *et al.* 2014).

However a number of hepcidin isoforms have been reported from various fishes, most of them are cultured or freshwater fishes of economic importance. Reports of hepcidin isoforms from wild fishes are scanty. This chapter deals with the molecular and functional characterization of the two hepcidin isoforms obtained from two fishes of exclusively marine origin. One of the fish is a non-acanthopterygian deep sea fish of primitive taxa and the other one is an acanthopterygian fish of monophyletic origin.

4.2 Materials and Methods

4.2.1 Sample collection

Live sample of *Chlorophthalmus bicornis* were collected from a depth of 500 m off Andaman Sea using a High Speed Demersal Trawl (HSDT) net operated on-board FORV *Sagar Sampada* (Ministry of Earth

Sciences, Govt. of India) during Cruise No. 292. Gills were dissected out from freshly caught fish and preserved in 100% methanol at the biological laboratory on-board the research vessel. Live Moorish idol was obtained as a by-catch from 30 m depth zone off Cochin coast during daily fishing cruise of FORV *Sagar Sakthi* (Central Institute of Fishery Technology, Kochi). The live specimen was brought to the laboratory and sacrificed humanely. Gills were carefully dissected out immediately after the death of the animal and transferred to TRI® reagent. Samples preserved in TRI® reagent were stored at -80 °C until use.

4.2.2 Total RNA extraction

Total RNA was extracted from the samples preserved in TRI® reagent following the manufacturer's instruction. The purity and quality of RNA was tested on 0.8% agarose gel. For details on RNA extraction procedure refer Chapter 2 section 2.2.4.

4.2.3 cDNA Synthesis

Single stranded cDNA was synthesized from RNA (RNA having an absorbance ratio of ≥1.8 at A_{260}/A_{280}) using reverse transcription in a 20 µl reaction mixture containing 5 µg total RNA. For details on cDNA synthesis refer section 2.2.6 of Chapter 2.

4.2.4 PCR amplification and cDNA cloning

The PCR amplification of hepcidin like AMPs from cDNA of fishes were performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase (Fermentas, Inc.). The

primers Hepcidin F (5'- CGAAGCAGTCAAACCCTCCTAAGATG-3') and Hepcidin R (5'- GAACCTGCAGCAGACACCACAT CCG-3') (Ren *et al.* 2006) were used for the amplification. The PCR condition involved an initial denaturation of 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s 60 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gel, stained with SYBR® Safe and visualized under UV light using the Gel Doc XR system and the quantity one programme (Bio-Rad). Positive reactions were identified. PCR products were purified and ligated into cloning vector.

4.2.4.1 Ligation

The PCR products were cloned onto the pGEM-T Easy vector (Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2x), 0.5 µl of the vector (50 ng/µl), 3.5 µl PCR product (600 ng/µl) and 1 µl of T4 DNA ligase (3 U/µl). The ligation mix was mixed well by repeated pipetting and was incubated at 22 °C for 1 h.

4.2.4.2 Preparation of competent cells

For transformation of pGEM-T Easy vector with the insert, *E. coli* DH5α was used as the cloning host. Competent cells of *E. coli* DH5α were prepared as follows. Briefly, a single colony of *E. coli* DH5α was inoculated to 10 ml sterile LB broth and allowed to grow overnight at 37 °C with shaking at 150 rpm. An aliquot of 5 ml overnight culture was then inoculated into 50 ml fresh sterile LB broth and again incubated for 2 h at 37 °C with shaking at 250 rpm to get cells in log phase. The culture was centrifuged at 6000 rpm for 20 min at 4 °C and the cells were

harvested by decanting the supernatant. The pelletized cells were then re-suspended in 100 mM $CaCl_2$ by gentle vortexing. The mixture was placed on ice for 45 min with intermittent swirling and mixing and centrifuged at 6000 rpm for 20 min at 4 °C. The supernatant was decanted and cell pellet was re-suspended again in 1 ml of 100 mM $CaCl_2$. The resulting competent cells were aliquoted into suitable storage vials with 15% glycerol and stored at -80 °C until use.

4.2.4.3 Transformation

Recombinant plasmids were transformed to *E. coli* DH5α competent cells using heat-shock method. In brief, 10 µl of the ligated product was transferred to 50 µl of competent cells in a polypropylene tube on ice and mixed well by gentle flicking. The mixture was then incubated on ice for 20 min. After the incubation, the cells were then given a heat-shock at 42 °C for 45 s in a water bath. The time and temperature should be maintained exactly, to facilitate effective transformation. Then the tubes were immediately returned to ice for 2 min. To this, 550 µl SOC medium at room-temperature was added and incubated for 1.5 h at 37 °C with shaking at 250 rpm. The transformation mixture (300 µl) was then spread onto duplicate LB/IPTG (100 mM)/X-gal (80 µg/ml)/ampicillin (100 µg/ml) agar plates. The plates were then incubated overnight at 37 °C. The transformed colonies were selected based on blue/white colony screening. The white colonies were selected and patched on to LB/Ampicillin, X-gal/IPTG plates and incubated overnight at 37 °C.

4.2.4.4 Confirmation of the presence of insert by colony PCR

Each individually patched white colonies were further screened by colony PCR using vector specific primers *viz*., T7 and SP6 as well as gene specific primers to confirm the presence of the insert DNA in the transformed cells. Colony PCR was performed in a 25 µl reaction mix containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 µM dNTPs, 0.4 µM each primer and 1U Taq DNA polymerase. Thermal profile used for vector specific amplification was an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 57 °C for 20 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Thermal profile used for the amplification of gene specific was same as above profile except for annealing temperature. Annealing was done at 60 °C for gene specific primers. PCR products were then analyzed on 0.8% agarose gel electrophoresis and visualized using Gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA). The clones with the required size of inserts were identified by colony PCR and segregated for further use.

4.2.4.5 Plasmid extraction and purification

Positive colonies with required insert size were inoculated to 10 ml LB/Ampicillin (100 µg μl^{-1}) broth and incubated overnight at 37 °C with shaking at 250 rpm. Plasmid with the insert was then extracted and purified using GenElute HP' plasmid MiniPrep kit (Sigma) following manufacturer's protocol. Briefly, 2 ml of overnight transformed *E. coli* culture was transferred to 2 ml micro centrifuge tubes (MCT) and the cells were harvested by centrifugation at 12000 x g. The pellet was then re-suspended in 200 µl

resuspension solution complemented with RNase. To the cell suspension, 200 µl of the lysis buffer was added to lyse the cells. The content was then mixed immediately by gentle inversion until the mixture becomes clear and viscous. The cell debris was then precipitated by adding 350 µl of the neutralization/binding solution, mixed well by gentle inversion and centrifuged at 12000 x g for 10 min to remove the cell debris, proteins, SDS and chromosomal DNA as a viscous precipitate. Columns were prepared by inserting a GenElute HP MiniPrep binding column into the provided 1.5 ml tubes. About 500 µl of the column preparation solution was then added to the MiniPrep column and centrifuged at 12000 x g for 1 min. The flow through liquid was discarded and the cleared lysate obtained in the previous step was then transferred to the prewashed column and the flow through was discarded. The column was washed twice with wash solution I and II respectively. An additional centrifugation of the empty column was also done at maximum speed for 1-2 min, to remove excess ethanol. The column was then transferred to fresh collection tubes supplied with the kit and Plasmid DNA was eluted with 100 µl of elution solution (10 mM Tris-HCl) by centrifuging at 12000 x g for 1 min. The plasmid DNA present in the elute was stored at -20 °C until use. Plasmid DNA thus obtained was analyzed and confirmed by agarose gel electrophoresis. The plasmids with insert were sent for sequencing at SciGenom, Kochi, India.

4.2.5 Sequence Analysis and Molecular Characterization

The sequences were analysed, trimmed and assembled using GeneTool software. The cDNA-based gene sequences were translated using Expert Protein Analysis System (ExPASy). Homology searches of

nucleotide sequence and the deduced amino acid sequence were performed using BLASTn and BLASTp algorithm of the NCBI. Peptide characterization and further analysis of the amino acid sequence was performed using various computer based algorithms as explained in section 2.2.10 of Chapter 2.

4.2.6 Phylogenetic Analysis

The relevant preprohepcidin sequences of fishes as well as representatives from other vertebrate classes were retrieved from GenBank and multi aligned using ClustalW and GeneDoc computer programmes. Phylogenetic analysis was carried out using MEGA version 5.05 and the phylogenetic tree was constructed by Neighbor Joining (NJ) method with complete deletion of gaps and subjected to 1000 repetitions of bootstrap. A distance matrix was also constructed to analyze the evolutionary distance of fish hepcidins to previously reported hepcidins.

4.3 Results

Two cDNA fragments, a 294 bp fragment (Fig. 4.1) with an ORF of 87 aa and a 267 bp fragment with an ORF of 81 amino acids have been identified from the gill tissues of deep sea fish Spiny-jaw green- eye, *C. bicornis* (Fig. 4.2) and Moorish idol, *Z. cornutus* (Fig. 4.3) respectively by RT-PCR. BLAST analysis of the nucleotide and deduced amino acid sequences revealed that the peptides belonged to hepcidin super family of antimicrobial peptides. The obtained nucleotide sequences and deduced amino acid sequences of both peptides are shown in Fig. 4.4. Both nucleotide and amino acid sequences of the peptides were deposited in GenBank database (GenBank Accession number: JX163299 and JX163300 for *C. bicornis* and *Z. cornutus* respectively).

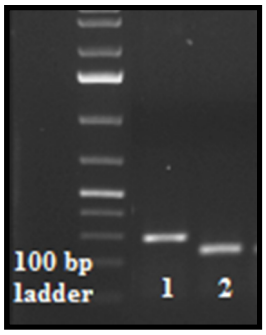


Fig. 4.1: Approximately 300 bp amplicon obtained from the fishes *C. bicornis* (Lane 1) and *Z. cornutus* (Lane 2) with the primer Hepcidin 1

Fig. 4.2: *Chlorophthalmus bicornis*

Fig. 4.3: *Zanclus cornutus*

(a)

```
atgaagactttcagggttacagtagcagtggccgtcatgctcacctttatttgtattcag
 M  K  T  F  R  V  T  V  A  V  A  V  M  L  T  F  I  C  I  Q
gagagctctgctggtccagtctctgaagtacaagagctggaggagccaatgaacaatgac
 E  S  S  S  A  G  P  V  S  E  V  Q  E  L  E  E  P  M  N  N  D
aatccagttgttgtgcatgaagagatgtcagaagaatcctggaagatgccgtataacaga
 N  P  V  V  V  H  E  E  M  S  E  E  S  W  K  M  P  Y  N  R
cagaagcgtaaccctgctggctgtaggttttgctgtggttgctgtcccaacatgcgcgga
 Q  K  R  N  P  A  G  C  R  F  C  C  G  C  C  P  N  M  R  G
Tgtggtgtctgctgcaagttctga
 C  G  V  C  C  K  F  *
```

(b)

```
atgaagacattcagtgttgcagtggccgtcgtgctcacctttatttgccttcagcagagctctgct
 M  K  T  F  S  V  A  V  A  V  V  L  T  F  I  C  L  Q  Q  S  S  A
gtcccagtcactgaaggggaagatccagaggtgccaatggtggatgtatatgaagaggttccagtg
 V  P  V  T  E  G  E  D  P  E  V  P  M  V  D  V  Y  E  E  V  P  V
gagtcgtggaagatgccgtataacaacagacttaagcgcagtgctgctggctgtaaattttgctgc
 E  S  W  K  M  P  Y  N  N  R  L  K  R  S  A  A  G  C  K  F  C  C
ggttgctgtcctgacatgaacggatgtggtgtctgctgcaggttctga
 G  C  C  P  D  M  N  G  C  G  V  C  C  R  F  *
```

Fig.4.4: Nucleotide and deduced amino acid sequence of the hepcidin isoform from the gill mRNA transcripts of *C. bicornis* (a) (GenBank ID: JX163299) and *Z. cornutus* (b) (GenBank ID: JX163300). Bold underlined portion specifies the 22 amino acid signal peptide. The bioactive mature peptide is highlighted in grey, followed by the stop codon indicated with asterisk.

4.3.1 Hepcidin from Spiny-jaw Green-eye (*Chlorophthalmus bicornis*)

4.3.1.1 Sequence analysis

Similarity searches using BLAST algorithm indicated that the 87 amino acid preprohepcidin of *C. bicornis* showed 81% similarity with hepcidin 2 precursor of *Acanthopagrus schlegelii* (AS-hepc2) (**AY669377.2**) and 80% similarity with hepcidin-like precursor of *Pagrus major* (**AAS66305.1**). The softwares SignalP and Prop 1.0 were used to predict the cleavage sites for the enzymes Signal-peptidases and Pro-peptidases respectively. These cleavages will result in the formation of bioactive mature peptides from inactive preprohepcidin. SignalP software predicted a cleavage site for the 87 aa prohepcidin of *C. bicornis* between amino acids Ala^{24} and Gly^{25}, which will result in the formation of 24 aa signal peptide. Cleavage of propeptide convertase would result in the formation of 39 aa prodomain and a C-terminal 24 aa mature peptide. A characteristic core domain signature formed of 8 conserved cysteines could be observed in the mature peptide region of the peptide (Fig. 4.5). The 24 aa mature peptide of *C. bicornis* hepcidin will be referred here after as Hepc-CB1

4.3.1.2 Molecular characterization of Hepc-CB1

Peptide characterization using ProtParam and APD softwares indicated Hepc-CB1 to be cationic with a net charge of +3. Whereas prepropeptide as such and propeptide of *C. bicornis* hepcidin were found to be anionic. The 24 aa Hepc-CB1 was found to have a presumed molecular weight of 2.53 kDa and a theoretical isoelectric point (p*I*) of 8.54 as predicted by ProtParam. APD predicted its hydrophobic potential as 54%, which is contributed by amino acids Val (1), Phe (2), Cys (8), Met (1) and Ala (1). The 24 aa Hepc-CB1

mature peptide differs from its most similar counterpart, the mature peptide of AS-hepc2 sequence by two amino acids. The N-terminal amino acids Ser^{65} and C-terminal Arg^{87} of AS-hepc2 were replaced by the amino acids Asp^{65} and Lys^{86} in Hepc-CB1 respectively (http://aps.unmc. edu/AP/ main.php). The structural model created by the software ViewerLite 4.6 exhibits β-hairpin like structure for Hepc-CB1 (Fig. 4.6 a), consisting of two antiparallel beta sheets strengthened by four disulphide bonds. The disulphide bonds were formed between Cys^{68}–Cys^{85}, Cys^{71}–Cys^{84}, Cys^{72}–Cys^{81}, and Cys^{74}–Cys^{75}. The β1 sheet composed of amino acids from Arg^{69} to Cys^{71} and β2 sheets composed of amino acids Gly^{82} to Cys^{84}.

4.3.2 Hepcidin from Moorish idol (*Zanclus cornutus*)

4.3.2.1 Sequence analysis

In BLAST analysis preprohepcidin of *Z. cornutus* exhibited 70% similarity with hepcidin-like precursor of *Pagrus major* (**AAS66305.1**), 69% similarity with hepcidin 2 (**AAU00795.1)** and 67% similarity with hepcidin 4 (**AAU00797.1**) precursors of *Acanthopagrus schlegelii*. SignalP software has recognized a signal peptidase site at position 22 between the amino acids Ala^{22} and Val^{23}. A propeptide cleavage site for the enzyme pro-peptide convertase was also predicted by software ProP 1.0 after the motif RX (K/R) R. Cleavage of propeptide convertase would result in the formation of a 35 aa prodomain and a 24 aa C-terminal mature peptide. Similar to *C. bicornis* hepcidin an 8 cysteine core domain signature could be observed in the mature peptide region of *Z. cornutus* hepcidin also (Fig. 4.5). The 24 aa mature hepcidin of *Z. cornutus* will be referred here after as Zc-Hepc1.

4.3.1.2 Molecular characterization of Zc-Hepc1

Peptide characterization using ProtParam and APD softwares showed that Zc-Hepc1 has a predicted molecular weight of 2.43 kDa, a net positive charge of +1 and an overall hydrophobic index of 58%. Apart from the mature peptide, prepropeptide as a whole and propeptide of *Z. cornutus* hepcidin were found to be anionic. According to APD analysis Zc-Hepc1 exhibited maximum similarity to the mature hepcidin of *A. schlegelli* (As-hepc2). Nevertheless, Zc-Hepc1 has showed difference in 4 aa to that of As-hepc2. The amino acids Pro^{66}, Arg^{70}, Asn^{78} and Arg^{80} of the As-hepc2 are replaced by the amino acids Ala^{59}, Lys^{63}, Asp^{71} and Asn^{73} in Zc-Hepc1 resulting in a difference of 16.67%. The structural models created by ViewerLite 4.2 demonstrate a β-hairpin like structure for Zc-Hepc1 (Fig. 4.6 b), formed of two antiparallel beta sheets strengthened by four disulphide bonds. The disulphide bonds were formed between Cys^{62}–Cys^{79}, Cys^{65}–Cys^{78}, Cys^{66}–Cys^{75}, and Cys^{68}–Cys^{69}. The β1 sheet is framed by the amino acids Lys^{63}, Phe^{64} and Cys^{65} while the amino acids Gly^{76}, Val^{77} and Cys^{78} constituted β2 sheet.

4.3.3 Phylogenetic analysis

The phylogenetic tree constructed based on the alignment of preprohepcidins of *C. bicornis* and *Z. cornutus* with previously reported hepcidins composed of two main clusters (Fig. 4.7). Cluster 1 was framed entirely of fish hepcidins, whereas cluster two was of non-fish vertebrates and which remained as an out group to cluster 1 formed of fishes. The main cluster, cluster 1 could be clearly demarcated into two sub clusters structured by HAMP1 and HAMP2-like hepcidins.

The HAMP2-like cluster could again be subdivided into three small clusters. *viz.*, cluster 1) formed of hepcidin isoforms from *Acanthopagrus schlegelli*, 2) formed of hepcidins characterized from the genus *Epinephelus* and 3) cluster formed by Antarctic notothenioid fishes. According to the distance matrix calculated using MEGA 5.05, the fish hepcidins were related more closely to the hepcidin isoforms of *Morone chrysops* and *Dicetrachus labrax* followed by the hepcidins of *Lateolabrax japonicus*, *A. schlegelli* and *Oreochromis niloticus*. Hepcidin of *Solea senegalensis, Paralichthys olivaceus and Psetta maxima* represent the most distant lineage of *C. bicornis* and *Z. cornutus* hepcidin and are of HAMP 1-like peptides also.

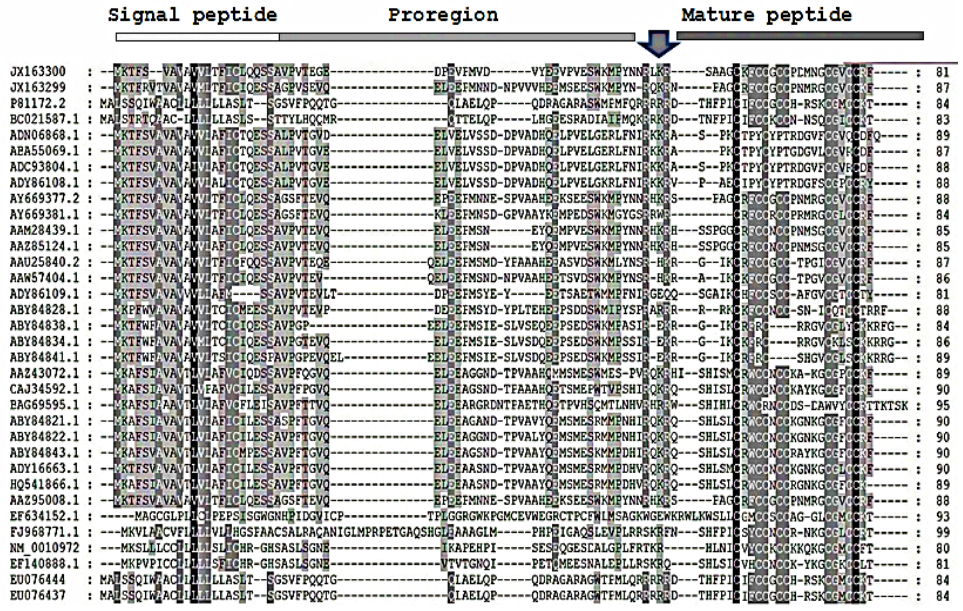


Fig. 4.5: ClustalW multiple alignment of *C. bicornis* and *Z. cornutus* hepcidin with other reported fish and non-fish vertebrate hepcidins created using GeneDoc version 2.7.0. Signal peptide, propeptide and mature peptide is marked. The motif for propeptide convertase RXK/RR motif which is typical of the propeptide convertase is marked with bold down arrow.

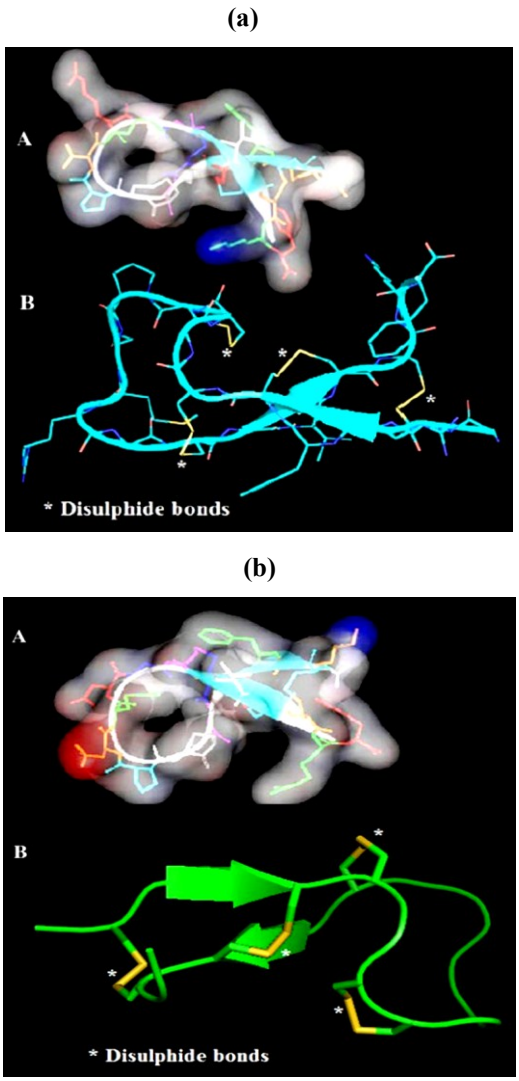


Fig. 4.6: Spatial structure of Hepc-CB1 (a) and Zc-Hepc1 (b) created with the software ViewerLite version 4.2 using the pdb data generated by SWISS-MODEL server. The crystal structure of hybrid white striped bass hepcidin (PBD ID: 1S6W) was used as template for the data generation. The spatial structure (A) and the diagrammatic representation of the β-hairpin structure (B) are presented in figure. The disulphide bonds which stabilize β- hairpin are highlighted with asterisks (*)

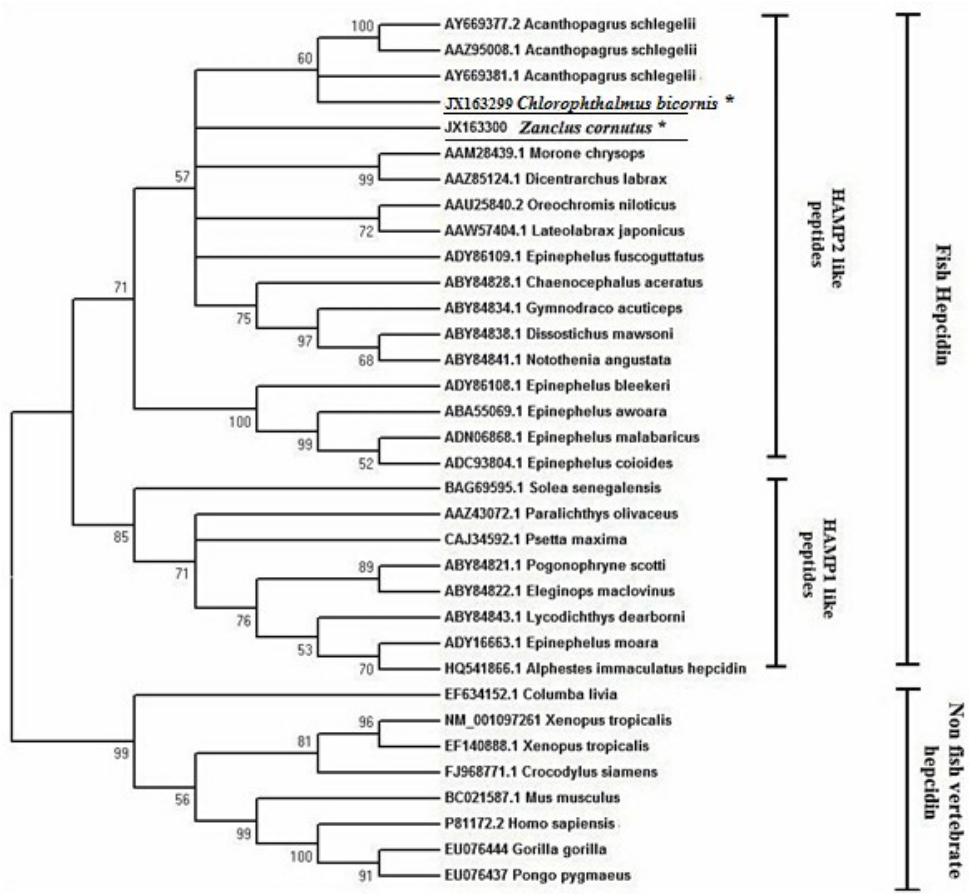


Fig. 4.7: A bootstrapped neighbour joining tree constructed using MEGA version 5.05 illustrating the phylogenetic relationship of *C. bicornis* and *Z. cornutus* hepcidins with previously reported hepcidin like antimicrobial peptides of fishes and non-fish vertebrates. Numbers on the branch indicates the percentage of 1000 bootstrap samples.

4.4 Discussion

Hepcidin is an acute-phase protein, endogenously synthesized as an inactive precursor and is known to participate in host defense of almost all vertebrates. It is one among most studied antimicrobial peptides from fishes (Masso-Silva *et al.* 2011; Rajanbabu and Chen 2011b), mainly

because of its physiological relevance as antimicrobial peptide and iron regulating hormone (Shi and Camus 2006). Hepcidin can directly bind to iron-binding proteins like ferroprotein, which leads to its internalization and degradation resulting in limited availability of iron for bacterial growth (Falzacappa and Muckenthaler2005; Barnes *et al.* 2011). The iron regulatory activity of Hepcidin thereby further enhances its antimicrobial activity. Though the first report of hepcidin was from mammals, adaptive evolution of hepcidin gene is prominent in fishes; since mammals (except mice) possess a single copy of HAMP1 gene and fishes have more than two copies of hepcidin genes (Hilton and Lambert 2008). These multiples always fell in HAMP2 class, which were reported only in acanthopterygian till the identification of Hepc-CB1from *C. bicornis*.

The 87 and 81 amino acid preprohepcidin obtained from the gill mRNA transcripts of *C. bicornis* and *Z. cornutus* showed all the hallmarks of fish hepcidin with a more or less conserved leader signal peptide and a mature bioactive peptide preceded by a variable pro-region. The predicted signal peptides of the fishes were found to be similar to the signal peptides of most of the previously reported hepcidins. A 24 aa signal peptide was identified from *C. bicornis* hepcidin and is in agreement with previous reports but the predicted signal peptide of the preprohepcidins from *Z. cornutus* was only 22 aa in length. However, it was rich in hydrophobic amino acids like valine and alanine, and proceeded by a N-terminal lysine as in previous hepcidin reports. Cleavage of the signal peptide would result in the formation of a prohepcidin which is exported to the lumen of endoplasmic reticulum, where the prohepcidin undergoes a second cleavage probably by the action of furin and related propeptide convertases

like PC5, PC7 and PACE4 (Schranz *et al.* 2009; Singh *et al.* 2011) leading to consequential release of the mature bioactive peptide. These are serine proteases which play crucial role in the maturation of prohepcidin in mammals (Schranz *et al.* 2009). Propeptide convertase cleavage site in hepcidins is generally preceded by a conserved RXK/RR motif and its presence was observed in both *C. bicornis* and *Z. cornutus* hepcidins. The propensity of a cleavage site for pro-peptidases was predicted by the software ProP 01 also. Generally the function of propeptide region is to assist the transit of AMP through the subcellular compartments and to protect the cellular mechanism of host against toxic effects of mature peptide (Valore and Ganz 2008). The release of mature peptide from propeptide region of hepcidin is pretty fast, unlike other cysteine rich AMPs like defensins, indicating the efficiency of hepcidin in immune response (Valore and Ganz 2008).

Hepcidins of varying size (20-31 aa) have been reported from several fishes irrespective of their evolutionary position and ecological niches (Martin-Antonio *et al.* 2009; Yang *et al.* 2011). Among this 24 aa hepcidin is the most common. The mature hepcidins, Hepc-CB1 and Zc-Hepc1 were also 24 aa peptides. Hepc-CB1 and Zc-Hepc1 differ in 6 aa from each other and in 2 and 4 amino acids respectively from their most similar counterpart, As-hepc2. This diversity in the relatively conserved mature peptide region could be because of synonymous to non-synonymous substitution and positive Darwinian selection mediated adaptive evolution of hepcidins with respect to environmental cues (Padhi and Verghese 2007; Xu *et al.* 2008). Such adaptive evolution of hepcidin could also be observed in various groups of Antarctic notothenioid fishes where a novel

type of hepcidin isoform with only four cysteine residues was also observed (Xu *et al.* 2008). Cold oxygen-rich water could be the cue factor of hepcidin evolution in Antarctic fishes (Xu *et al.* 2008). Same way, the evolution and diversification of hepcidins in fishes may be attributed to the austere and dynamic external environment within which they live.

The measure of cationicity is an important parameter in the determination of antimicrobial properties mediated by electrostatic attraction and subsequent cell lysis. Owing to the presence of three cationic amino acids (R, K) and absence of anionic amino acids, Hepc-CB1 was cationic enough (p*I* 10 +3) to get attracted to negatively charged target cell membranes. But, unlike previously reported hepcidins, cationicity of Zc- Hepc1 is limited to +1 only. The observed theoretical p*I* of Zc-Hepc1 is far less from the average p*I* of fish hepcidins and comparatively less than that of the lowest recorded p*I* of 7.70 for turbot hepcidin 2 (Pereiro *et al.* 2012). Apart from the highly conserved basic amino acid positions; no additional basic amino acids were present in Zc-Hepc1. Furthermore, acquired cationicity of +2 contributed by basic amino acids were diminished to +1 due to the presence of negatively charged aspartic acid (D). This is the reason for the reduced theoretical p*I* and cationicity (7.46 and +1) of the Zc-Hepc1 and it lies in the margin of acidic and basic nature. However, evidences approve participation of such hepcidin isoforms as in Atlantic salmon and turbot (p*I* 7.73 and 7.70 respectively) in pathogen-induced defense in teleost fishes (Douglas *et al.* 2003a; Pereiro *et al.* 2012). Thus the hepcidins, Hepc-CB1 and Zc-Hepc1 gratifies all the basic essential qualification to function as an AMP. They are low molecular weight, (2.53 and 2.43 kDa respecively) amphipathic (hydrophobicity of

53 and 58%) peptides that could form β-hairpin like structures on membrane mimetic environments. It is evident from the secondary structure predicted by the software PyMol, that the bonding pattern (C^1-C^8,C^2-C^7,C^3-C^6 and C^4-C^5) of the hepcidins, Hepc-CB1 and Zc-Hepc1 were similar to the hepcidins reported from human, sea bass and black porgy (Hunter *et al.* 2002; Lauth *et al.* 2005; Yang *et al.* 2011). The vicinal disulphide bonds formed at the hairpin turn (C^4-$C^{5)}$ could be the possible decisive domain for biological activity of the molecule (Kemna *et al.* 2008). Conservation of basic structure with modified physicochemical properties from lower vertebrates to higher vertebrates signify the evolution of iron regulatory peptide from a protein ancestor designed to participate in host immune defense (Shi and Camus 2006).

Phylogenetic analysis of the amino acid sequences of hepcidins identified from *C. bicornis* and *Z. cornutus* with previously reported vertebrate hepcidins disclose orthologous origin of fish and non-fish vertebrate hepcidins. Yet, an obvious divergent evolutionary pattern of fish hepcidin isoforms from rest of the vertebrates is being represented clearly in the cladogram. Evolution and diversification of hepcidin gene is more prominent in fishes (Hilton and Lambert 2008) than any other vertebrates due to the varied ecological niches to which they adapt and highly dynamic austere external environment. Two main lineages of HAMP gene, HAMP1 and HAMP2-like groups of peptides were observed in fishes (Hilton and Lambert 2008) and it is well shown in the phylogenetic tree. In the hepcidins identified from *C. bicornis* and *Z. cornutus* the motif QSHLS/DTHFP was absent at the N-terminus of the mature peptide. While considering the charge distribution on preprohepcidin, only the mature

bioactive peptide was found to be cationic. The prepropeptide and the propeptide were anionic in nature. From the above facts it is obvious that the hepcidin isoforms identified from the fishes belonged to HAMP2-like lineage and is further confirmed by the phylogenetic analysis as they were grouped together with cluster, fish HAMP2-like peptides.

In the phylogenetic tree, hepcidin isoform identified from *Z. cornutus* was placed in a midway between two groups of perciform fishes, the sea bream and sea bass. Though the family Zanclidae is a monophyletic group, it certainly comes under the order Perciformes. Hence the positioning could be justified. But, placement of hepcidin identified from a non-acanthopterygian fish *C. bicornis*, in the clade structured of hepcidins characterized from perciform fishes was quite surprising. *C. bicornis* belonged to the monophyletic order, Aulopiformes and paraphyletic family, Chorophthalmidae coming under Protacanthopterygii and is much primitive in evolutionary position than perciform fishes, probably evolutionarily the most advanced order of fishes. Such type of grouping may not necessarily be because of the direct evolutionary relationships of *C. bicornis* with these fishes but may be due to the absence of hepcidins sequences already identified and deposited in GenBank that connect these groups together.

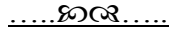
To date HAMP2-like lineage had been characterized from acanthopterygian fishes only (Hilton and Lambert 2008). This is the first report of a HAMP2-like peptide from a non-acanthopterygian deep sea fish, *C. bicornis*. Existence of two lineages of HAMPs is a result of mass gene duplication events happened over a few million years time period.

Evidence portrayed that adaptive evolution and diversification of HAMP2-like peptides in fishes is due to positive Darwinian selection (Padhi and Verghese 2007). The extreme habitats of the deep sea might have produced fascinating evolutionary events (Davis 2010) like gene duplication and conservation of HAMP2 like genes in non-acanthopterygian fishes also. More investigation is required in this context to get a clear picture of the molecular evolution and functional diversification of HAMP2 like genes in deep-sea fishes, which is living in an environment where heterotrophy dominates.

The hepcidin isoform of *Z. cornutus* is definitely a new subtype of HAMP2 like lineage. The exact biological function of the peptide is yet to be revealed. However, previous reports of up-regulation of low cationic hepcidin transcripts during pathogenic invasion suggests defensive role of these peptides in host immune responses (Douglas *et al.* 2003a; Pereiro *et al.* 2012). The HAMP2-like group is generally considered as AMPs and HAMP1-like group as an iron regulating hormone as well as antimicrobial in function. However even without the iron regulatory motif, sea bass hepcidin transcripts were found to be up regulated with iron overload (Rodrigues *et al.* 2006). In humans rather than iron regulatory function, hepcidin upregulation and down regulation was observed with several physiological as well as pathological responses. Recently Na^+ dependent active transport of hepcidin across the cell membrane has been reported. It was the first report of an AMP that is being transported actively (Chothe *et al.* 2011). Systemic inflammation mediated upregulation and increased serum hepcidin level in patients with chronic obstructive pulmonary disease was also observed (Tandara *et al.* 2015). Likewise, though

hepcidin is one of the most studied iron regulatory antimicrobial peptide; exact physiological function of all hepcidin isoforms are yet to be understood.

This is the first report of novel HAMP2-like peptides from a non-acanthopterygian fish *C. bicornis* and acanthopterygian perciform coral fish Z. *cornutus*, which may provide useful information to the distribution of HAMP2-like genes in non-acanthopterygian fishes, its molecular evolution and phylogenetic relationships. High similarities of both the fish hepcidins in physico-chemical properties with other hepcidins of proven antimicrobial activity strongly endorse it to be an antimicrobial peptide. Exact physiological function of hepcidin besides antimicrobial and iron regulatory properties is yet to be characterized. Further studies on the antimicrobial activity of Hepc-CB1 and Zc-Hepc1 through solid phase synthesis or recombinant expression would reveal the potentials of these new hepcidin isoforms as a possible therapeutic agent in aquaculture/ medicine. Moreover this hepatic antimicrobial peptide would definitely be a valuable addition to AMP repository.



Chapter 5

MOLECULAR CLONING, RECOMBINANT EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF HEPCIDIN (Zc-Hepc1)

Contents

5.1 Introduction

Antimicrobial peptides are small, low molecular weight host defense peptides with a wide spectrum of activity against invading microorganisms including Gram-positive and negative bacteria, fungi, parasites and viruses (Boman 2003; Hancock and Lehrer 1998) and has recently been emerged as novel antimicrobial agents for use in therapeutics, animal drugs and food preservatives (Wei *et al.* 2005). Most of the AMPs exhibit encouraging results against multiple drug resistant (MDR) bacteria which have been identified as a serious socio-economic concern and global public health issue of the current era. Besides promising antimicrobial property, many of the AMPs could resist proliferation and development of neoplastic cells (Hancock and Sahl 2006; Hoskin and Ramamoorthy 2008). Furthermore, owing to the peculiar mechanism of action and relatively fewer propensities to develop resistance against bacteria, these peptides are in the

prime arena of scientific and industrial research (Rajanbabu and Chen 2011a; Seo *et al.* 2012).

Despite all the promising features of AMPs; before recommending as a candidate drug, certain fundamental issues like biological activity, exact mechanism of action, efficacy and safety have to be addressed and resolved. For this purpose, extensive structural and functional characterization are essential (Li 2011). Readily available large quantity of pure and active AMPs in a cost effective way is the prime requisite to advance peptide-mediated chemotherapy and is the major constraint in the existing scenario. The three possible ways to obtain peptides are natural isolation, solid-phase peptide synthesis and recombinant expression (Li 2011). Isolation of peptides from natural sources is a time consuming and tedious process which requires source organism in large quantities. It is practically impossible as well as highly detrimental to the ecosystem and environment (Li and Chen 2008). Nevertheless, solid phase synthesis by chemical methods is a widely used method; the complexity and high cost make it less advantageous. Furthermore improper folding and secondary structure formation limit its use in advanced research since secondary structure is the crucial element in determining the activity of the peptide (Andersson *et al*. 2000). Therefore, heterologous expression of AMPs in a suitable host is the only possible cost effective way to obtain AMPs in huge quantities (Ingham and Moore 2007).

Hepcidin is a small cysteine-rich β-sheet peptide with multiple biological functions and (Krause *et al.* 2000). It is distributed widely among vertebrates from fishes to mammals (Hilton and Lambert 2008). Despite intensive research on structure and biological function of hepcidin,

the exact physiological role and mechanism of action of various hepcidin isoforms are yet to be discovered. To acquire required quantity of hepcidin isoforms for further research to decipher the ambiguity in hepcidin functions neither natural extraction nor solid-phase chemical synthesis could be employed as *in vitro* disulphide bond formation by oxidation is a pretty complex task (Wu *et al*. 2003; Klüver *et al*. 2006). Recombinant expression in prokaryotic/eukaryotic/baculoviral expression systems are well characterized and is the sole possible way to produce AMPs in a relatively cost effective method (Baneyx 1999; Bryksa *et al.* 2006; Rashid *et al.* 2009; Vidovic *et al.* 2009).

Escherichia coli is considered as the most appropriate host for *in vivo* heterologous expression of AMPs to-date because of its ability to grow rapidly at high density in relatively inexpensive medium. Furthermore, its genes are fairly well characterized and a number of highly efficient genetically-modified strains are easily available (Baneyx 1999; Vidovic *et al.* 2009). However, recombinant expression in *E. coli* often faced failure due to vector instability and imminent toxicity of the expressed gene of interest to the host (Vidovic *et al.* 2009). Besides, the small size and high cationicity make them more susceptible to host proteolytic enzymes (Li 2011). To circumvent the lethal effects of expressed peptides against the host, AMPs are often expressed as fusion proteins, which will mimic the peptide's precursor structure and protects the host as the propeptide does in peptide biosynthesis (Vassilevski *et al*. 2008). Thioredoxin and glutathione transferase (GST) are the two most frequently used fusion tags in recombinant expression of AMPs (Li 2009). Recently a small ubiquitin-related modifier (SUMO) tag is also being used as a new

fusion carrier with improved solubility and folding capacity of the target proteins (Butt *et al*. 2005; Malakhov *et al*. 2004). S•Tag™, T7•Tag®, GST•Tag™, His•Tag®, HSV•Tag® and Nus•Tag™ are some of the commonly used fusion tags. Besides neutralizing toxicity, fusion tags also facilitate increased solubility and easy detection of target protein. Some fusion tags like GST also serves as an affinity tag for the purification of expressed peptide (Li 2011). Hexa Histidine-Tag is the most common affinity tag used (Li *et al.* 2006). Instead of the solubility increasing fusion tags, those with the tendency to form inclusion bodies are also used to express highly toxic peptides. Because insoluble expression is more efficient in masking the peptide's toxic effect against host cell (Vidovic *et al*. 2009; Zorko and Jerala 2010). Fusion carriers like PurF fragment (16.3 kDa), ketosteroid isomerase (13.5 kDa), PaP3.30 (17.6 kDa) and TAF12 histone fold domain (8.4 kDa) are some of the commonly used insoluble expression tags (Lee *et al*. 2000; Majerle *et al*. 2000; Rao *et al*. 2004). Inteins, protein counterparts of introns also used as fusion tags owing to their self-cleaving and ligation capacity. Usage of exogenous enzymes/ chemicals to cleave off fusion tags from the target protein could be limited by using intein tags and is an advantage over the rest of the fusion tags (Perler *et al*. 1994; Xu and Evans 2003). Enterokinase, factor Xa and thrombin are the three most widely used enzymes for tag removal (Li 2011).

Appropriate vector and host selection are crucial for the success of recombinant expression. *E. coli* is the most common host used for expression of AMPs till-date. Yeast expression system, *Saccharomyces cerevisiae* or *Pichia pastoris* and mammalian cell culture system with Chinese hamster ovary (CHO) are also used pretty widely (Andersen and

Krummen 2002; Koliaraki *et al.* 2008). Care should be taken while selecting prokaryotic expression system because native AMPs often contain codons that are rarely used in *E. coli*. Recombinant expression in such host will result in poor yield (Li 2011). Following are some of the commonly used plasmid-host combinations used by various scientists. pET-32c (+) expression vector in *E. coli* strain BL21 trxB (DE3) (Yang *et al.* 2004), pET-23d/YUH plasmid in Rosetta (DE3) pLysS (Moon *et al.* 2007), pNM726, pNM850 and pSB1001 in *E. coli* Tuner (DE3) cells (Srinivasulu *et al.* 2008), pGEX-3X (Amersham Biosciences) and *E. coli* BL21(DE3) (Zhang *et al.* 2005), pET32a (+) in *E. coli* C43 (DE3) (Tian *et al.* 2009) pET-28a+ vector in *E. coli* BL21 (DE3) pLysS cells (Cai *et al.* 2012), pET-28a in *E. coli* BL21 (DE3) pLysS (Haidong *et al.* 2013).

Hepcidin (Zhang *et al.* 2005; Wallace *et al.* 2006; Koliaraki *et al.* 2008; Rashid *et al.* 2009; Cai *et al.* 2012; Hao *et al.* 2012; Haidong *et al.* 2013; Janakiraman *et al.* 2015), pleurocidin (Brocal *et al.* 2006), piscidin (Moon *et al.* 2007), parasin (Zhao *et al.* 2015), adenoregulin (Cao *et al.* 2005), arencin (Ovchinnikova *et al.* 2007), gallinacin (Ma *et al.* 2008), ASABF (Zhang *et al.* 2000), carcinin (Brockton *et al.* 2007), melittin (Lazarev *et al.* 2007), cecropin (Xu *et al*. 2007), cathelicidin-LL37 (Yang *et al.* 2004), penaeidin (Destoumieux *et al.* 1999), magainin (Jin *et al.* 2006), antilipopolysaccharide factor (Somboonwiwat *et al.* 2005; Yedery and Reddy 2009) and SpStrongylocins (Li *et al.* 2010) are some of the recombinantly expressed and functionally charcterised AMPs.

The chapter deals with recombinant expression and functional characterisation Zc-Hepc1, a 24 aa mature hepcidin identified from coral

fish Moorish idol (*Zanclus cornutus*) using prokaryotic expression system. Zc-Hepc1 is a 8 cysteine, 4 disulphide bonded β-sheet peptide of comparatively less cationicity (+1). Cationicity is a crucial factor in detemining biological activity of AMPs. Hence *in vitro* functional characterization of Zc-Hepc1 which is a relatively a less cationic peptide is essential to delineate its primary role in the host defense of fishes.

5.2 Materials and methods

5.2.1 Target AMP Zc-Hepc1

A novel hepcidin isoform identified from the coral reef fish Moorish idol (*Zanclus cornutus*) was selected for molecular cloning and recombinant expression analysis (Fig. 5.1). The preprohepcidin identified from the fish was 81 aa in length. The 24 mer mature peptide region (Zc-Hepc1) alone was selected for recombinant expression and further characterization. Zc-Hepc1 was a 24 aa peptide with 4 cysteine disulphide bond core domain signature, which allowed it to form a β-hairpin like structure. Zc-Hepc1was found to be a slightly cationic, amphipathic small peptide with a net positive charge of +1, hydrophobic index of 58% and a predicted molecular weight of 2.43 kDa. For details refer chapter 4.

```
atgaagacattcagtgttgcagtggccgtcgtgctcacctttatttgccttcagcagagctctgct
 M  K  T  F  S  V  A  V  A  V  V  L  T  F  I  C  L  Q  Q  S  S  A
gtcccagtcactgaaggggaagatccagaggtgccaatggtggatgtatatgaagaggttccagtg
 V  P  V  T  E  G  E  D  P  E  V  P  M  V  D  V  Y  E  E  V  P  V
gagtcgtggaagatgccgtataacaacagacttaagcgcagtgctgctggctgtaaattttgctgc
 E  S  W  K  M  P  Y  N  N  R  L  K  R  S  A  A  G  C  K  F  C  C
ggttgctgtcctgacatgaacggatgtggtgtctgctgcaggttctga
 G  C  C  P  D  M  N  G  C  G  V  C  C  R  F  *
```

Fig. 5.1: Nucleotide and deduced amino acid sequence of *Z. cornutus* Hepcidin

5.2.2 Designing primers with restriction sites

The active mature peptide region, Zc-Hepc 1 was selectively amplified from the preprohepcidin identified from *Z. cornutus* using specially designed primers with restriction sites and Histidine tag. Restriction sites for *Nco* I and *Hind* III were added to forward and reverse primer respectively. Additionally a histidine tag was also added to the forward primer. HMI- Forward: 5' CCATGGGCCATCATCATCATCATCATAGTGCTGCTGGCTGTAA 3', HMI Reverse: 5' CGAAGCTTTCAGAACCTGCAGCAGAC 3' were the primers used for amplification (Fig. 5.2).

F-CATGCCATGG GC CATCATCATCATCATCAT AGTGCTGCTGGCTGTAA

R- CG AAGCTT TCAGAACCTGCAGCAGAC

Fig. 5.2: Diagrammatic and verbal representation of specially designed primers used to amplify mature hepcidin Zc-Hepc1 from preprohepcidin for recombinant expression. Forward and Reverse primers with restriction site and His-tag was shown in figure. Blue coloured sequences represent His-tag and red colour sequence represents restriction sites for the enzymes *Nco* I and *Hind* III.

5.2.3 PCR Amplification of the target peptide.

Target peptide was amplified using specially designed primers with restriction sites from the plasmids (Refer section 4.2.4.5 of Chapter 4 for more details). PCR amplification of target peptide was performed in a 25 µl reaction volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM each primer 1U Taq DNA polymerase and 1 µl plasmid as template. The thermal

profile used for the PCR amplification was 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 30 s and a final extension at 68 °C for 10 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light using Gel Doc™ XR+ imaging system (Bio-Rad, USA).

5.2.4 Cloning of the target peptide gene into pGEM®-T easy vectors

The PCR products were cloned into pGEM-T easy vector as explained in section 4.2.4 of Chapter 4 and the recombinant plasmids were used for transforming to *E. coli* DH5α cells using heat shock method. Transformed cells were plated on to LB/ ampicillin/X-gal/IPTG plates and plasmids were isolated from positive colonies (for details refer section 4.2.4 of chapter 4). Plasmids were sequenced at SciGenom sequencing facility Kakkanad, Kochi. Sequence identity was confirmed before proceeding with further experiments.

5.2.5 Restriction digestion

The recombinant plasmids (pGEM-T Zc-Hepc1) were digested with restriction enzymes *Nco* I and *Hind* III (FastDigest restriction enzymes, Thermo). For the purpose, 100 µl of purified plasmid was incubated with 10 µl of reaction buffer and 1 unit of each enzyme, *Nco*1 and *Hind* III for 1 h at 37 °C followed by an inactivation at 65 °C for 20 min. Similarly expression vector into which target gene to be incorporated (pET32a+) was also digested using the same enzymes to get complimentary sticky ends for the construction of recombinant plasmid. Digested plasmids were loaded onto 1% agarose gel to confirm digestion.

5.2.6 Gel elution of restriction digested insert and expression vector

The restriction digested plasmids were loaded onto 1.2% agarose gel (25 µl per well) and the released fragments were gel purified using GenJET™ Gel Extraction Kit (Thermo Scientific, USA) following manufacturer's instructions. Briefly, the gel containing desired DNA fragment was excised with a clean scalpel and transferred to a pre-weighed 1.5 ml vial. Gel was excised as close to the DNA band as possible to minimize the gel volume. Then the binding buffer was added (100 µl for 100 mg agarose gel) and the gel mixture was then incubated at 50-60 °C for 10 min or until the gel gets dissolved fully. For DNA fragments ≤500 bp, 1:2 volume of isopropanol (100 µl for 100 mg agarose gel in binding buffer) was added and mixed well by vortexing. From the solubilized gel solution, 800 µl was loaded on to pre activated column and centrifuged at 12,000 x g for 1 min. To the column, 700 µl wash solution was added and centrifuged at 12000 x g for 1 min. The flow through was discarded in both the steps. The empty column was further centrifuged at 12000 x g for 1 min to ensure complete removal of residual wash buffer. Column was then placed in a fresh collection vial (2 ml) and added 50 µl of elution buffer (10 mM Tris-HCl, pH 9.0). Centrifuged at 12,000 x g for 1 min and the eluted purified DNA was stored at -20 ºC until use. The concentration of DNA was measured spectrophotometrically at 260/280 nm in a UV-VIS Spectrophotometer (U2800, Hitachi, Japan).

5.2.7 Construction of recombinant expression vector (pET32a+) and transformation into *E. coli* DH5α

Purified restriction digested target gene was ligated to similarly digested pET32a+ vector (Novagen, UK) by following the manufacturer's

instructions. Briefly, 10 µl ligation mixture containing 1 µl pET32a+ vector (50 ng μl^{-1}), 4 µl target gene, 1 µl ligation buffer (10x), 1 µl T4 DNA ligase (1U μl^{-1}) and 3 µl MilliQ were incubated at 4 °C overnight. The ligated products were then transformed to *E. coli* DH5α competent cells as described in Chapter 4 Section 4.2.4.3.

5.2.8 Confirmation of insert by colony PCR

Positive clones were selected and patched on to LB/ampicillin (100 µg μl^{-1}) plates and all the isolated colonies were subjected to colony PCR using vector specific (T7 forward and reverse) as well as gene specific primers (HMI- forward and reverse). White colonies (template) picked from the transformed plates were dispensed into the PCR reaction mix (25 µl) containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 200 µM dNTPs, 0.4 µM each primer (T7 forward and T7 reverse) and 1U Taq DNA polymerase. The thermal profile used was an initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 15 sec, annealing at 52 °C for 20 s and extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. PCR products were then analyzed on 1.5% agarose gel electrophoresis, visualized and documented using Gel Doc™ XR Gel documentation system (Bio-Rad, USA). For colony PCR using sequence specific primers, annealing temperature was 60 °C. Rest of the cycle was similar as above described.

5.2.9 Plasmid extraction and sequencing.

Transformed cells were multiplied in LB/ampicillin broth (Incubation at 37 °C overnight with shaking at 250 rpm). Plasmid was extracted from the freshly cultured cells as described in section 4.2.4.5 of chapter 4. Purified

plasmids were analyzed on 1.2% agarose gel. Purity and size of the plasmid was confirmed and sent for sequencing at the SciGenom sequencing facility, Kochi. In-frame confirmation of the genes with the ATG of the pET32a+ system was further done with Gene Tool software.

5.2.10 Transformation of Expression Host *E. coli* Rosetta-gami™ B (DE3) pLysS.

5.2.10.1 Selection of expression host

Rosetta-gami™ B (DE3) pLysS is a highly efficient and ideal expression host to use with pET 32 vectors. It is a derivative of original strain Rosetta-gami™ B with combined expression features of the strains BL21, Origami and Rosetta. The strain is specially designed to express eukaryotic proteins that contain codons rarely used in *E. coli*. Rosetta strain supplies tRNAs for these rare codons; AUA, AGG, AGA, CUA, CCC, and GGA on a compatible chloramphenicol-resistant plasmid, pRARE under the control of their native promotor. Mutation in the Thioredoxin reductase (*trxB*) and Glutathione reductase (*gor*) genes greatly enhance cytoplasmic disulphide bond formation. Likewise, the strain is deficient in *lac* permease gene (*lacY*) and *lon, ompT* proteases. The former allows adjustable, inducer (IPTG)-dependent and uniform expression of target protein throughout all cells in a culture and the latter help to reduce proteolytic degradation of the expressed protein. Strains having the designation (DE3) pLysS are lysogenic for a λ prophage that contains an IPTG inducible T7 RNA polymerase gene and a pACYC184-derived plasmid that encodes T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase. T7 lysozyme represses basal expression of target genes under the control of the T7 promoter.

5.2.10.2 Preparation of competent Rosetta-gamiTM B (DE3) pLysS cells

Competent Rosetta-gami^{TM} B (DE3) pLysS cells were used for the study (Novagen). Competent cells were prepared. For the purpose, Rosetta-gami^{TM} B (DE3) pLysS cells were streaked on LB agar plate containing the antibiotics kanamycin (15 µg/µl), chloramphenicol (125 µg/µl) and tetracycline (340 µg/µl). Single colony was then inoculated in 10 ml LB medium containing the entire antibiotic described above and grown overnight at 37 °C with shaking at 150 rpm. An aliquot of 5 ml of overnight culture was then inoculated into 50 ml LB antibiotic broth and incubated at 37 °C for 2 h at 250 rpm to get cells in their log phase. The culture was centrifuged at 6000 rpm for 20 min at 4 °C to harvest the cells. To the harvested cells, 0.1 M $CaCl_2$ (1/4th original culture volume) was added and the pellets were re-suspended. The re-suspended cells were placed in ice for 45 min with intermittent swirling and mixing. The cells were centrifuged at 6000 rpm for 20 min at 4 °C, supernatant was decanted and again the cell pellets were re-suspended in 1 ml of 0.1 M $CaCl_2$. The competent cells thus prepared were stored at -80 °C with addition of 10-15% glycerol until use.

5.2.10.3 Transformation to expression host

Recombinant pET32a+ plasmids were transformed to competent Rosetta-gami^{TM} B (DE3) pLysS competent cells using heat shock method as described in section 4.2.4.3 of chapter 4. The transformation mixture (300 µl) was plated on to LB agar plates, containing the antibiotics ampicillin (100 µg/ml), kanamycin (15 µg/ µl) chloramphenicol (125 µg/µl) and tetracycline (340 µg/µl). The plates were then incubated overnight and

observed for colonies. Individual colonies thus obtained were screened for a final round with vector specific as well as gene specific primers.

5.2.11 Standardization and recombinant expression of hepcidin in Rosetta-gami™ B (DE3) pLysS cells.

A single colony of Rosetta-gami™ B (DE3) pLysS cells with recombinant pET32a+ construct containing 'Zc-Hepc1' was inoculated into 5 ml LB broth supplemented with antibiotics viz., ampicillin (100 µg /µl), Kanamycin (15µg/µl) cholramphenicol (125 µg/µl) and Tetracycline (340 µg/µl) and incubated at 37 °C and shaking at 250 rpm overnight. One ml of the overnight culture was then added to 100 ml LB broth supplemented with above listed antibiotics and further incubated at 37 °C for 3-4 h until OD_{600} reaches 0.6. Then cells were induced with 0.1 mM IPTG. Prior to induction with IPTG, 5 ml culture was aliquoted and kept separate, which served as the un-induced control. An un-induced culture also maintained in 100 ml to know the basal level expression. Both the cultures (induced and uninduced) were incubated further for 5-7 h at 37 °C with shaking at 250 rpm. A 2 ml aliquot was removed from the induced culture at every 1 hour interval and continued up-to 7th hour to identify time period required for maximum expression. The 2 ml culture aliquots were then centrifuged at 12000 x g for 2-5 min at room temperature to get the cells pelletized and the pellets were stored at -20 °C.

5.2.12 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE) analysis of cell pellets

Hour-wise recombinant protein expression was analyzed by SDS- PAGE analysis. Small amount of pellet from un-induced control and hour-wise sampling were boiled in 10 µl sample buffer [150 mM Tris-Cl,

pH 7, 12% SDS, 30% glycerol, 6% mercaptoethanol, 5% coomassie brilliant blue R-250 (CBB)] for 15 min. A short spin was given to the boiled samples and the supernatant was subjected to 16% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Expression was analysed on a 4% stacking and 16% resolving (running) gel. Entire operation was carried out using '4-gel Mini-PROTEAN® Tetra cell protein electrophoresis unit, BioRad, USA'. Tris (pH 8.9) and Tris-tricine (pH 8.3) were used as anode and cathode buffer respectively. A voltage of 50 and 120 V was provided to stack and separate the sample respectively. When the indicator dye, CBB traversed out of the gel, the apparatus was switched off, plates were separated and the stacking gel alone was taken out carefully. Then the gel was stained in CBB R-250 (0.5% CBB R-250, 40% methanol and 10% acetic acid in distilled water) and de-stained in de-staining solution (10% methanol and 10% acetic acid in distilled water) and photographed using Gel-DOC™ XR+ imaging system (BioRad, USA).

5.2.13 Production of recombinant hepcidin (Zc-Hepc1)

Production of recombinant hepcidin Zc-Hepc1 was done in Rosetta-gami™ B (DE3) pLysS cells as described in section 5.2.12. A total of 1L culture was produced in four 250 ml conical flasks. The culture was induced with 0.1mM IPTG when OD_{600} reached 0.6. Cells were harvested by centrifugation at 12000 x g for 2-5 min at room temperature after 4 hr incubation at 37 °C and shaking at 250 rpm. The pellets were stored at -20 °C for further analysis.

5.2.14 Purification of the recombinant 6x-His tagged protein using Ni-NTA spin column.

Purification of the 6x-histidine tagged recombinant protein was carried out with Ni-NTA spin column (Qiagen®) according to the manufacturer's protocol. Briefly, the cells (Cell pellets from ≈ 5 ml culture) were allowed to thaw for 15 min at room temperature and re-suspended in 700 µl of buffer B (7 M Urea, 100 mM Na_2HPO_4, pH 8.0). The cell suspension was kept at room temperature for 15 min with continuous agitation. The solution will become translucent once the lysis is complete. When the solution became translucent, the lysate was centrifuged at 12000 x *g* for 15-30 min at room temperature to pellet the cellular debris. Supernatant is collected and stored for further analysis.

Prior to loading of cleared lysate, the spin column was equilibrated with buffer B. For the purpose, 600 µl of buffer B was loaded into the spin column and centrifuged at 2900 rpm for 2 min with lid open to ensure complete removal of the buffer. To the pre-equilibrated column 600 µl of clear lysate supernatant with 6x-Histidine tags was loaded and centrifuged at 1600 rpm and room temperature for 5 min. An additional centrifugation of 5 min was also provided as the lysate was highly viscous. The flow through was collected and saved for SDS-PAGE analysis to test the efficiency of binding. Then the column was washed with 600 µl buffer C (8 M Urea, 100 mM NaH_2PO_4, pH 6.3) to remove excess and untagged proteins. For washing, the spin column was centrifuged at 2900 rpm for 2 min at room temperature. Again the flow through was collected for SDS-PAGE analysis to examine the stringency of washing. Washing step was repeated to get pure protein. The recombinant protein was then eluted

twice with 100 µl of buffer E (8 M urea, 100 mM NaH_2PO_4, pH 4.5) by centrifugation at 2900 rpm in room temperature. The whole process was repeated many times to purify the whole pellet obtained from 1L culture. The eluted purified protein was pooled and stored at -20 °C for further use.

5.2.15 Concentrating and refolding the eluted peptides

The Ni-NTA spin column eluted 6x-Histidine tagged protein was further concentrated to one tenth using Amicon Ultra Cenrifugal 3 kDa cut-off Membranes (Millipore). An initial centrifugation of 5000 x g for 30 mins was performed to get the sample concentrated. Then the concentrated samples were reconstituted to the original volume using the refolding buffer (Tris-Cl pH 8- 50 mM, EDTA- 0.1 mM and NaCl- 0.15 M) and centrifuged at 5000 x g for 30 mins. Centrifugation/washing was repeated for 10-12 times with the refolding buffer to remove the denaturing salts (urea). Meanwhile the recombinant protein will refold to its native form as it is re-suspended in refolding solution. After complete removal of urea from the sample, the concentrated sample was diluted to desired volume in refolding buffer.

5.2.16 Enterokinase digestion of recombinant fusion protein.

The enzyme Enterokinase (from porcine intestine (E0885-40UN) supplied by Sigma) was used to cleave and release the peptide of interest, Zc-Hepc1 from the fusion protein. For the purpose, to 1 ml of refolded proteins, 2 units of enterokinase was added and incubated at 37 °C for 14-16 h. Enterokinase treated sample was analyzed on SDS-PAGE to check the efficiency of enterokinase as well as to confirm the size of the peptide.

5.2.17 Purification of recombinant AMP, Zc-Hepc1

The enterokinase cleaved the fusion protein into two unequal fragments, a relatively large fragment of ≈18 kDa and a small fragment of ≈2.4 kDa; the latter being the recombinantly expressed peptide, Zc-Hepc1. The enterokinase digested sample was centrifuged in Amicon Ultra-Centrifugal 3 kDa cut-off membrane (Millipore) at 5000 x g for 30 min. Zc-Hepc1 having lower molecular weight will pass through the filter and was collected at the bottom of the tube, while high molecular weight fusion protein will be retained by the filter. The collected filtrate was transferred to fresh storage vials and stored at -20 °C for further analysis. Refolding of the peptide was done as described earlier (section 5.2.15). Since the EDTA in refolding buffer interferes with antibacterial assays, it was replaced with 50 mM Tris-HCl.

5.2.18 Quantification of recombinant AMP, Zc-Hepc1

The recombinant hepcidin, Zc-Hepc1 was quantified with Quant-iTTM protein assay kit using Qubit fluorometer (Invitrogen, UK). Accuracy of the kit is reliable and protein-to-protein variation is very less. The Qubit fluorometer will give values for Quant-iT protein assay kit in µg/ml. The Quant-iT working solution was prepared by diluting the Quant-iT protein reagent in Quant-iT protein buffer (1:200) and mixed well without any air bubbles. From the working solution 190 µl was aliquoted into a fresh clean 0.5 ml tube. An aliquot of 10 µl recombinant peptide was added to the tubes and mixed by mild vortexing to avoid air bubbles (air bubbles cause error in the readings). The mixture was incubated for 15 min at room temperature and then measured using the Quant-iT protein

programme. The assays were performed at room temperature and the signal was stable for 3 h. The sample concentration was calculated using the following equation:

Concentration of sample = QF value x (200/X)

Where, QF value = the value given by the Qubit fluorometer,

X = volume of sample (in microliters) added to the assay tube

5.2.19 Antibacterial assay

5.2.19.1 Bacterial strains used for assay

Antimicrobial activity of the recombinant peptide was tested against both Gram-positive and Gram-negative bacteria. *Bacillus cereus* (MCCB 101) and *Staphylococcus aureus* (MTCC 3061) were the Gram-positive bacteria used. Five strains of Gram-negative bacteria, *viz.*, *Vibrio parahaemolyticus* (MCCB 133), *V. cholerae* (MCCB 129), *Edwardsiella tarda* (MTCC 2400), *Aeromonas hydrophila* (MCCB 113) and *Pseudomonas aeruginosa* (MCCB 119) were tested.

5.2.19.2 Preparation of bacterial suspension

Pure strains of bacteria were streaked onto sterile nutrient agar slants and incubated 18-24 h at optimum temperature for each bacterium. Sufficient quantity of 50 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) was added to the culture slants and the bacterial cells were scraped off using sterile inoculation loop. The bacterial suspension was then transferred to sterile fresh tubes and mixed well for uniformity. Optical Density (OD) was measured at 600 nm and the cell

density was adjusted to 10^3 cells/10 µl of bacterial suspension depending upon OD measurements.

5.2.19.3 Liquid growth inhibition assay

Antibacterial assay of recombinant Zc-Hepc1 was carried out using Liquid Growth Inhibition assay in 96 well, microwell plates as described by Huang *et al*. (2006) with modifications. To test antimicrobial activity 6 dilutions of the peptide was prepared ranging from 0.16 to 5.21 µM and assay was carried out in triplicates. Briefly, 10 µl of bacterial suspension was mixed with 10 µl of serially diluted recombinant peptides. The assay was carried out with positive and negative controls. Ampicillin (1mg/ml) and 50 mM Tris-HCl were used as positive and negative controls respectively. Controls were prepared in a similar way to that of test samples. A blank control was also maintained similarly by mixing 10 µl of the HEPES buffer with 10 µl of bacterial suspension (10^3cells/ ml). The whole assay set including blank and controls were incubated at room temperature for 2 h. After incubation, 80 µl of the nutrient broth was added to each well and again incubated approximately for 5 to 6 h at 37 ^oC. Subsequently 25 µl solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 µg/ml) was added to each well and incubated on a shaker at room temperature for 30 minutes. The reaction was stopped by adding 125 µl of acidified isopropanol (0.1 N HCl in isopropanol) and was incubated on a shaker at room temperature for 30 minutes for the complete dissolution of formazan dye. Absorbance was measured at a wavelength of 570 nm with 630 nm as reference using a Microplate reader (Tecan, USA). Bacterial mitochondrial

dehydrogenase will reduce MTT to the purple formazan dye and hence colour intensity is directly proportional to number of live cells. Percentage of growth inhibition (%) was calculated from the OD measurements as follows:

$$\text{Inhibition \%} = 100 - \text{Growth \%}.$$

Where, Growth % = [OD test well (wells with bacteria + peptides)/ OD of Negative control] × 100.

The Minimum Inhibition Concentration (MIC) of recombinant Zc-Hepc1 was estimated by Probit analysis using PriProbit computer based program.

5.3 Results

5.3.1 PCR amplification and cloning of target peptide, Zc-Hepc1

The 24 aa mature peptide region (Fig. 5.3), Zc-Hepc1 was specifically amplified from the full length preprohepcidin identified from the fish Moorish Idol using restriction primers. As a result, approximately 110 bp amplicon was obtained (Fig. 5.4a). The PCR product was purified and cloned into pGEM®-T Easy cloning vectors and transformed into *E. coli* DH5α competent cells. Colony PCR was performed using gene specific and vector-specific primers to confirm the presence of plasmid with insert. Approximately 110 bp amplicon was obtained with sequence-specific primers whereas approximately 230 bp length amplicon was obtained with vector specific primers (Fig. 5.4b).

MKTFSVAVAVVLTFICLQQSSAVPVTEGEDPEVPMVDVYEEVPVESWKMPYNNRLKR
SAAGCKFCCGCCPDMNGCGVCCRF

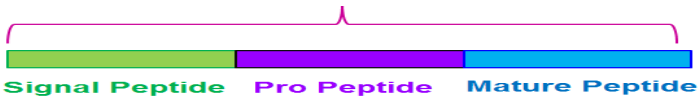


Fig. 5.3: The 81 aa hepcidin prepropeptide identified from the fish *Z. cornutus*. Signal peptide is marked in green, propeptide in purple and mature bioactive peptide in blue colors. Propeptide convertase processing site is marked in red. Conserved cysteines in the mature peptide region is marked in pink

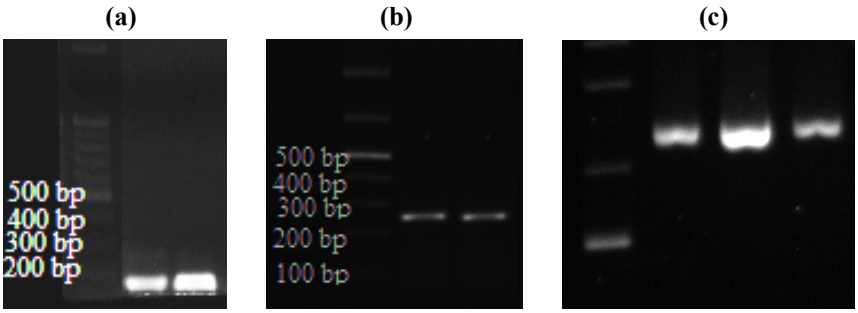


Fig. 5.4: (a) PCR amplification of Zc-Hepc1 with restriction primers. Lane 1 denotes 100 bp marker, Lane 2 and 3 shows 110bp amplicon of Zc-Hepc1.
(b) PCR amplification (colony PCR) Zc-Hepc1 insert in pGEMT vector construct using T7 forward and SP6 reverse primers. Lane 1 denotes 100 bp marker, Lane 2 and 3 shows 226 bp amplicon of Zc-Hepc1 (110 insert + 126 vector sequence = 226 bp)
(c) Agarose gel image of pGEMT easy cloning vector (Plasmid) with insert extracted from *E.coli* DH5α

5.3.2 Plasmid isolation, restriction digestion and cloning into pET32a+ vector

Plasmids were isolated from the transformed *E.coli* DH5α cells with desired plasmids propagated in LB/ampicillin broth. Purity of the isolated plasmids was confirmed by spectrophotometry and agarose gel electrophoresis (Fig. 5.4c). A DNA to protein ratio of >1.8 could be

observed in spectrophotometry for the isolated plasmid. The plasmids were sequenced and the result was analyzed by various online bioinformatic tools. Sequence was observed for frame shifts if any and no frame shifts were observed. Both restriction sites and His-tags were in correct frame and position. After affirming sequence frame, the plasmids with insert were digested with specific restriction enzymes, *Nco*1 and *Hind* III. Simultaneously, the vector pET32a+ was also digested with same enzymes. To confirm the digestion efficiency, both the plasmids with insert and vector was analyzed on agarose gel electrophoresis. Two distinct bands, one at the bottom of the gel (≈100 bp) and another one at the top of the gel (≈1500 bp) representing His-tagged Zc-Hepc1 and cut plasmid respectively could be observed from the gel image of restriction digested plasmid with insert (Fig. 5.5a). Likewise, the cutting efficiency and preciseness of pET32a+ also could be confirmed through gel imaging by its size and position when compared to the control plasmid (Fig. 5.5b). The restriction digested Zc-Hepc1 insert was then ligated to the hitherto digested pET32a+ vector using T4 DNA ligases and transformed to an intermediate host, *E. coli* DH5α to get high copy number.

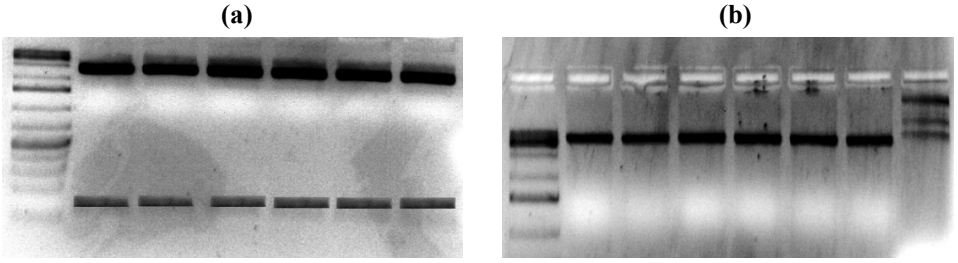


Fig. 5.5: Agarose gel image of recombinant plasmid digested with *Nco*1 and *Hind* III restriction enzymes. (a) Digested pGEMT vector and released Zc-Hepc1 insert (110 bp); (b) Digested pET32a+with *Nco*1 and *Hind* III enzymes

5.3.3 Transformation and recombinant expression in Rosetta-gami^{TM}B (DE3) pLysS competent cell

Plasmids were isolated from *E. coli* DH5α and transformed to competent Rosetta-gami^{TM}B (DE3) pLysS cells. Only transformed Rosetta-gami^{TM}B (DE3) pLysS cells with pET32a+ plasmid could grow on the medium containing the antibiotics ampicillin, kanamycin, tetracycline and chloramphenicol. Thus, the transformed cells were selected and subjected to a standardization of expression to know the optimum time taken for maximum expression. Sample aliquots were taken in 1 h intervals starting from 0 hour. There was no basal level expression at 0 h. The intensity of expression increased from the time of induction with IPTG (0.1 mM) to a maximum by 4 h, then reached a plateau (Fig. 5.6). Hence incubation time for mass production is selected as 4 h and mass production of recombinant Zc-Hepc1 in transformed Rosetta-gami^{TM}B (DE3) pLysS was carried out at 37 °C, with shaking at 250 rpm for 4 h. After culture the period, the cells were harvested and stored at -20 °C.

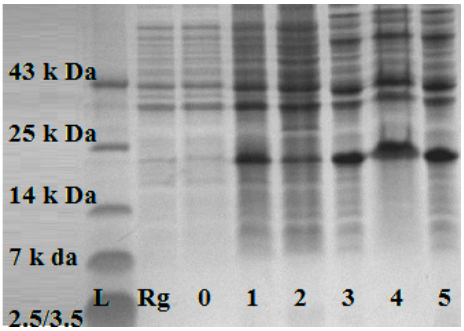


Fig. 5.6: SDS-PAGE analysis of the recombinant expression of Zc-Hepc1 in Rosetta-gami^{TM} B (DE3) pLysS, L- ladder, Rg- Rosetta-gami without pET32a+ vector, lane 0-5 represents recombinant expression after induction with 0.1M IPTG from 0 hour to 5th hour. At 4th hour maximum expression could be observed.

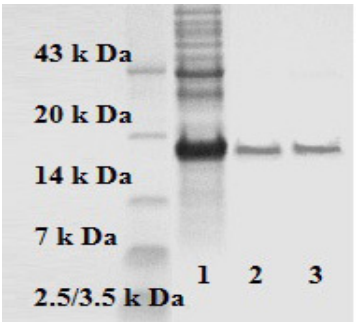


Fig. 5.7: SDS-PAGE analysis of crude and purified bacterial protein extract. Lane 1-Crude extract, 2-3 Ni-NTA spin column purified extract.

5.3.4 Purification and quantification or recombinant Zc-Hepc1

The 6x histidine tagged recombinant fusion protein was purified using Ni-NTA spin columns. A fusion protein of ≈20.43kDa was obtained from the cell lysate of Rosetta-gami^{TM}B (DE3) pLysS (Fig. 5.7). The peptide of interest was further cleaved off from the fusion protein with the enzyme Enterokinase and was separated by using Amicon ultra centrifuge 3 kDa cut off membrane. Efficiency of the cleavage was analyzed by SDS-PAGE and the cleavage is clearly visible in gel image (Fig. 5.8).

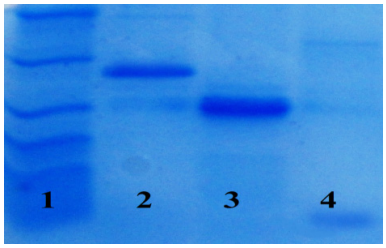


Fig. 5.8: SDS-PAGE analysis of recombinant fusion peptide and Enterokinase digested peptide. Lane1- Ladder, 2- Recombinant fusion peptide, 3- Fusion tag from which Zc-Hepc1 is released, 4-Released peptide

5.3.5 Antibacterial assay of recombinant Zc-Hepc1.

Antibacterial activity of recombinant Zc-Hepc1 was tested against both Gram-positive and Gram-negative bacteria using liquid growth inhibition assay. Out of seven strains of bacteria tested, Zc-Hepc1 exhibited antimicrobial activity against *Vibrio* sp. only. The peptide exhibited 82% and 70% reduction in growth of *Vibrio cholerae* (Fig. 5.9) and *V. parahaemolyticus* (Fig. 5.10) respectively at maximum tested concentration of the peptide (5.21µM). The peptide showed no or very poor microbicidal activity against rest of the tested microbes. Even at the highest tested concentration of the peptide, the activity against *P. aeruginosa* and

E. tarda (Table 5.1) was 6-9% only. It was relatively inactive against *Aeromonas hydrophila* as well as the tested Gram-positive bacteria. Promising results were shown against *V. cholerae* and *V. parahaemolyticus* only. Minimum inhibitory concentration (MIC) was calculated using 'probit' analysis. A MIC of 30.36 and 57.15 was obtained against *V. cholerae* and *V. parahaemolyticus* respectively.

Table 5.1: Antimicrobial activity of Zc-Hepc1 against different microorganisms

Con. Molarity (µM)	*S. aureus*	*V. cholerae*	*E. tarda*	*P. aeruginosa*	*V. para haemolyticus*	*A. hydrophila*
5.21	-	81.6	9	6	69.9	-
2.60	-	72.5	5	-	63.3	-
1.30	-	63.8	-	-	59.9	-
0.65	-	42.2	-	-	50.7	-
0.33	-	38.8	-	-	40.5	-
0.16	-	30.2	-	-	33.4	-
MIC	**-**	**30.36**	**-**	**-**	**57.15**	**-**

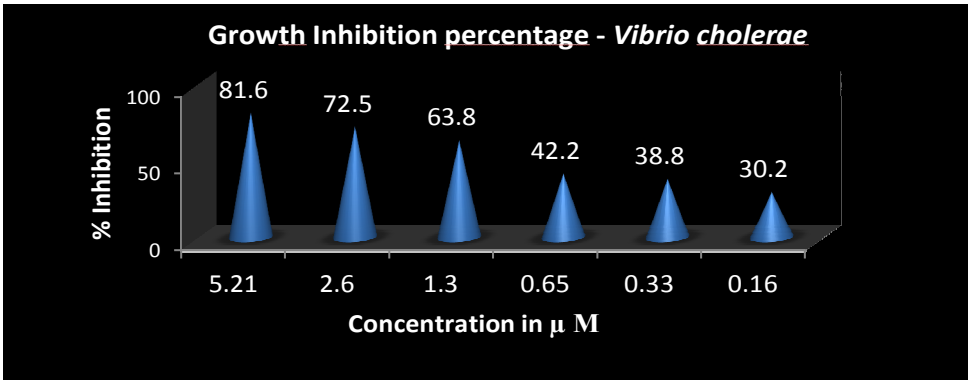


Fig. 5.9: Antimicrobial activity of Zc-Hepc1 against *V. cholerae*

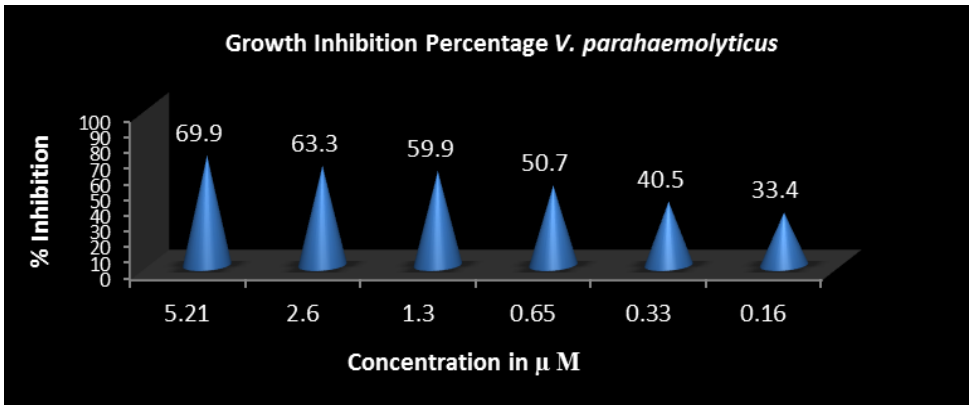


Fig. 5.10: Antimicrobial activity of Zc-Hepc1 against *V. parahaemolyticus*

5.4 Discussion

Investigations for novel therapeutic agents that could overcome bacterial resistance are progressing in an accelerated pace due to multiple drug resistant strategies of contagious bacterial pathogens to conventional antibiotics. Hepcidin is a relatively new member of AMP family with potential broad spectrum antimicrobial, antiviral and anti-cancer properties. It is widely distributed among vertebrates especially in fishes with numerous isoforms mainly falling into two classes, *viz*. HAMP 1 and 2. The exact biological function of all these isoforms is yet to be deciphered. The HAMP identified from the fish, Moorish idol is an exception from previously reported hepcidins due to its lower cationicity (+1) and intermediate theoretical p*I* (7.70). Molecular and functional characterizations of novel AMP isoforms like Zc-Hepc1 are essential to define the exact biological function of this peptide which makes them evolutionarily conserved as well as diversified concurrently.

Zc-Hepc1 is a short peptide with four disulphide bonds formed by 8 cysteines. Since the peptide is very short, chances of proteolytic degradation is very high and hence expressed as a fusion protein with thioredoxin tag (*trxB*), S•Tag and hexa-histidine tags (Vassilevski *et al.* 2008). The cloning vector used pET32a+ contains all the tags. These tags will safeguard the peptides from host proteolytic enzymes and mask its lethal effects to expression hosts by mimicking peptide's precursor structure (Li and Chen 2008). Some AMPs have failed to express in certain hosts due to codon incompatibility (Haidong *et al.* 2013) of the expression host with expressed protein. Rosetta-gami strains are designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli* (Brinkmann *et al*. 1989; Seidel *et al*. 1992; Kane 1995; Kurland and Gallant 1996) and are engineered with glutathione reductase (*gor*) and/or thioredoxin reductase (*trxB*) mutations that will greatly enhance the disulphide bond formation and solubility of the protein in *E. coli* cytoplasm (Prinz *et al*. 1997; Aslund *et al*. 1999). Hence Rosetta-gami™ B (DE3) pLysS strain was selected and used for heterologous expression to overcome rare codon bias and to obtain soluble proper folded disulphide bonded peptide in *E. coli* cytoplasm.

Transformed cells were induced with 0.1 mM IPTG and grown at 37 °C for 5-6 h. Expression was gradually increased from 1 hour onwards and peaked at 4th hour. IPTG concentration and temperature has significant role in success of expression (Li *et al.* 2014). Cells induced with low concentration of IPTG, in general, grow in much higher density and would yield better expression than cells induced with regular amount of IPTG (Schein and Noteborn 1988; Sorensen and Mortensen 2005). However an

IPTG concentration of 0.1 mM to 1 M could be seen in literature (Moon *et al.* 2007; Srinivasulu *et al.* 2008; Wang *et al.* 2009; Liang *et al.* 2013), comparatively lower concentration of IPTG (0.1 mM) was selected for the study because of the above reason. Furthermore, solubility of the expressed protein also affected adversely with increasing concentration of IPTG and temperature. At higher concentration of IPTG (1M) and elevated temperature (37 °C), hepcidin expressed as inclusion bodies (Zhang *et al.* 2005) have changed to soluble protein at 0.1 M IPTG and temperature 25 °C (Rabhi-Essafi *et al*. 2007). Recombinant Zc-Hepc1 would have expressed as a soluble protein in Rosetta-gami^{TM} B (DE3) pLysS cytoplasm owing to low concentration of inducer and the solubility increasing mutations specific to the host. Similarly, recombinant expression of hepcidin identified from Channel catfish liver in pET32a+ as thioredoxin fusion protein using *E. coli* BL21 (DE3) expressed as soluble protein in *E. coli* cytoplasm (Tao *et al.* 2014). Reasonable quantity of expressed protein could be observed in SDS-PAGE analysis without sonication and special treatment for inclusion bodies. Besides the usage as fusion tags to mask the deleterious effects of peptides to the host, hexa histidine tag and S•Tag could also be used for affinity purification using corresponding resins and buffers (Li 2011). The recombinant Zc-Hepc1 expressed as a fusion protein with His tag, trxB tag and S•Tag was purified using immobilized metal affinity chromatography on Ni-NTA spin columns under denaturing conditions. Ni-NTA spin columns effectively purified the His-tagged peptides from crude protein extract which was evident in SDS-PAGE analysis. After the purification, the denaturing agent, urea that is present in high concentration in the elution buffer was effectively replaced step by step

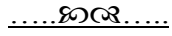
with 50 mM Tris-Cl solution using Amicon ultra centrifugal 3 kDa cut off membrane. This will in turn improve refolding of the peptide and could be observed by clearness of the solution (misfolded protein would precipitate in short time) (Tao *et al.* 2014). Fusion tags were later removed by digestion with enterokinase under optimum conditions as they may interfere with antimicrobial properties of the peptide owing to the net negative charge (Tao *et al.* 2014). Enterokinase is one among the most common enzymes used to cleave fusion partner from chimeric peptide (Zhou *et al*. 2007). The cleaved recombinant protein was then purified using Amicon Ultra-Centrifugal 3 kDa cut-off membrane (Millipore) and subjected to antimicrobial assay. Hepcidin in general is active against wide range of Gram-positive and Gram-negative bacteria, fungi, parasites and viruses (Chen *et al.* 2009a,b; Wang *et al.* 2010; Pan *et al.* 2011a,b; Masso-silva and Diamond 2014). Similarly, recombinant hepcidin also exhibited antimicrobial activity against both Gram-positive and Gram-negative bacteria. They were found to be active against Gram-positive bacteria such as *S. aureus, B. subtilis, B. megaterium, Corynebacterium glutamicum, Micrococcus lysodeikticus, M. luteus*, and *Listeria monocytogenes* in micro-molar range (Zhang *et al.* 2005; Wallace *et al.* 2006; Srinivasulu *et al.* 2008; Cai *et al.* 2012; Hao *et al.* 2012; Haidong *et al.* 2013; Lin *et al.* 2014; Tao *et al.* 2014; Janakiraman *et al.* 2015). Likewise Gram-negative bacteria *E. coli, A. hydrophila, A. sorbia, Pseudomonas aeruginosa, P. stutzeri, P. flourescens* and *Vibrio anguillarum* were also found to be inhibited by recombinant hepcidins identified from various fishes, humans and crocodile (Zhang *et al.* 2005; Wallace *et al.* 2006; Srinivasulu *et al.* 2008; Cai *et al.* 2012; Hao *et al.* 2012; Haidong *et al.* 2013; Lin *et al.* 2014; Tao *et al.*

2014; Janakiraman *et al.* 2015). Unlike previous reports, Zc-Hepc1 was insensitive towards tested Gram-positive bacteria (*S. aureus* and *B. subtilis*) and *A. hydrophila*. Very poor activity was exhibited against *E. tarda* and *P. aeruginosa*. Zc-Hepc1 is a relatively low cationic peptide when compared to those peptides which exhibited potential microbicidal activity against Gram-negative bacteria except Ec-hepcidin-3. This could be one possible reason for the lack of activity of Zc-Hepc1 against tested Gram-positive bacteria. Bioactivity of hepcidin is highly variable among diverse species and even between the isoforms of the same species (Yang *et al.* 2011; Cai *et al.* 2012). Hence concurrent inhibition pattern similar to previous reports could not be expected for a novel hepcidin isoform.

However anionic hepcidins (Orange spotted grouper hepcidin) and low cationic hepcidins (Orange spotted grouper and turbot) have been already identified, Zc-Hepc1 is the first 8 cysteine low cationic hepcidin (p*I* 7.70) to be characterized through recombinant expression. Ec-hepcidin 3 another low cationic hepcidin with theoretical p*I* 7.91 exhibited activity against both Gram-positive and Gram-negative bacteria (Haidong *et al.* 2013). Zc-Hepc1 is less cationic than Ec-hepcidin 3 and moreover Ec-hepcidin 3 is a four cysteine isoform. Hence comparison between two isoforms is meaningless as structural configuration and disulphide bonds are the two factors that determine the activity of the peptide (Papagianni 2003; Lin *et al.* 2014). Though Zc-Hepc1 exhibited no activity against Gram-positive bacteria, it exhibited promising activity against *V. cholerae* and *V. parahaemolyticus,* two important human pathogens. About 70-80% reduction of growth also could be observed at 5.21 µM concentration

against vibrios and hence it could be identified as antimicrobial agents with future prospective to develop as potential drugs against vibrios.

Hepcidin is proven as an antimicrobial agent beyond doubt. However, the exact mechanism of action of hepcidin is yet to be discovered. Some hepcidin isoforms like tilapia hepcidin 1-5, zebra fish hepcidin etc. are membrane lytic in nature and they inhibit bacterial growth by thinning of cell membrane and subsequent lysis. It is clearly evident from microscopy and light penetration assays also (Chang *et al*. 2011; Lin *et al*. 2014). Om-hep, another hepcidin isoform from Medaka exhibits an unknown mechanism of action apart from lytic mode of action. Similar to the activity spectrum of various hepcidin isoforms, mode of action of different isoforms vary considerably. The exact mechanism of action of hepcidin including Zc-Hepc1 has to be characterized substantially well to better understand its potential to develop as a novel antimicrobial drug.



Chapter 6

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF SYNTHETIC HEPCIDIN (HEPC-CB1)

Contents

6.1 Introduction

Emergence of Multiple Drug Resistant (MDR) pathogens is a serious health threat and socio economic concern of the present era. Indiscriminate and illegitimate use of antimicrobial drugs has resulted in MDR bacteria, fungi, parasites, viruses and even neoplastic cells (Narayana and Chen 2015). Microbial resistance is either the result of accumulation of resistance genes/plasmids towards specific drug or due to over-expression of efflux pumps that can effectively extrude wide range of drugs (Nikaido 2009). Antimicrobial peptides are new classes of antibiotics with potential to overwhelm bacterial resistance and replace conventional chemotherapeutic agents (Giuliani *et al.* 2007; Thundimadathil 2012). They are generally low molecular weight, small cationic peptides of less than 100 aa. More than 2400 peptides have been characterized to-date, out of which 90% are

cationic. Only 10% account for anionic peptides and these are represented by chromacins (Strub *et al.* 1996), temporins (Mangoni *et al.* 2005), dermcidins (Schittek *et al.* 2001) kappacins (Malkoski *et al.* 2001) Ec-hepcidins (Haidong *et al.* 2013) etc.

Antimicrobial peptides are highly diverse in amino acid composition, structural organization and mode of operation. Despite of structural diversity many of AMPs possess specific potential toxicity towards prokaryotic cells and relatively irresistible mechanism of action against them (Boman 2003). AMPs have exhibited promising results against not only bacteria but against fungi (Situ and Bobek 2000; Moerman *et al*. 2002), viruses (Wang *et al.* 2010), parasites (Gwadz *et al*. 1989; Wu *et al*. 2015). Besides antimicrobial properties, AMPs are also known to possess potent selective toxicity against neoplastic cells. Cancer is a leading cause of death worldwide representing $1/8^{th}$ of total mortality and about 7 million people die due to cancer and related diseases per year (Riedl *et al.* 2011; Thundimadathil 2012). Furthermore deleterious side effects of conventional chemotherapeutic agents due to inefficiency of these drugs to differentiate between neoplastic cells and actively dividing mammalian cells seek urgent attention to overcome the problem (Smith *et al*. 2000b; Cassidy and Misset 2002). Multiple drug resistance of neoplastic cells towards these commonly used chemotherapeutics worsens the situation to uncontrollable level. Therefore development of an improved anticancer drug with novel mechanism of action is needed urgently and AMPs are the most suitable candidates for the purpose (Hoskin and Ramamoorthy 2008; Riedl *et al.* 2011). All these features make AMPs as most promising candidates in

clinical and industrial research for the search of novel antibiotic agents (Marshall 2003; Giuliani *et al.* 2007).

Exact mechanism of action of AMPs always had been a controversy. However, membrane disruption and subsequent cell lysis is the most accepted and common method of action of AMPs (Boman 2003; Papagianni 2003). Besides, a complimentary mechanism targeting intracellular cascades also is well recognized (Boman *et al*. 1993; Cabiaux *et al*. 1994; Oh *et al*. 2000). Selective targeting of pathogens as well as neoplastic cells from normal cells is the key element that determines the efficiency of a drug. Microbial membranes and viral capsids are predominantly anionic in nature (Yount *et al.* 2006). Similarly neoplastic cells also have a net negative charge due to the presence of anionic membrane lipids on outer surface of the cell membrane (Reddy *et al.* 2004; Hoskin and Ramamoorthy 2008; Chen *et al.* 2014). Normal mammalian cell membrane is zwitter-ionic in nature due to the presence of cholesterol and special arrangement of anionic lipids on inner surface of the cell membrane (Chen *et al.* 2014). AMPs electrostatically get attached to negatively charged membranes and later destroy target cells either by membrane lysis/pore formation or by targeting intracellular machinery (Giuliani *et al.* 2007).

Hepcidin is cysteine-rich amphipathic peptide that contains multiple disulphide bonds. They are widely distributed among vertebrates (Shi and Camus 2006; Hilton and Lambert 2008), especially fishes and are among the most studied AMPs of fishes (Shi and Camus 2006). Multiple isoforms of hepcidin could be detected in fishes and the hepcidins differ from one another in amino acid composition and net charge. Biological function of

hepcidin isoforms has to be discovered for effective utilization of the peptide as novel antimicrobial agents. Large quantity of pure peptides should be readily available in a cost effective manner for the molecular and functional characterization of these potential future antibiotic molecules (Li 2011). Natural isolation from original source material, chemical synthesis and recombinant expression in suitable expression vectors and host are the three possible ways to get peptides of interest in large quantities (Li *et al.* 2006). Natural isolation is a time consuming, tedious and costly affair. Moreover the quantity yield is also very low. Recombinant expression is the cheapest and most reliable method to obtain peptides of desired quantity. Yet all peptides could not be produced by recombinant expression due to apparent toxicity of the peptide to the expression host and rare codon usage in AMPs (Li 2011; Haidong *et al.* 2013). Chemical synthesis of the peptide either through solid phase peptide synthesis or solution phase synthesis is a possible alternative. It is extremely useful in the production of toxic peptides. Furthermore, isotope labelling and amino acid modifications for *in situ* characterization of the peptides are possible only with chemical synthesis.

Recently functional characterization of various AMPs has been carried out with chemically synthesized peptides. Epinecidin (Yin *et al.* 2006), cathelicidin (Costa *et al.* 2011) piscidins (Peng *et al.* 2012), and defensin (Wang *et al.* 2012b) are some fish AMPs characterized through solid phase peptide synthesis. Antimicrobial activity of hepcidins identified from Hybrid striped bass (Lauth *et al.* 2005), tilapia (*Oreochromis mossambicus*) (Huang *et al.* 2007), Gilt headed sea bream (*Sparus aurata*) (Cuesta *et al.* 2008), Large yellow croaker (*Pseudosciaena crocea*) (Wang

et al. 2009), Black porgy (*Acanthopagrus schlegelli*) (Yang *et al.* 2011), Medaka (*Oryzias melastigmus*) (Cai *et al.* 2012), Turbot (*Scophthalmus maximus*) (Zhang *et al.* 2014), trout (*Oncorhynchus mykiss*) (Álvarez *et al.* 2014), Yellow croaker (*Larimichthys crocea*) (Li *et al.* 2014) and Chinese rare minnow (*Gobiocyprus rarus*) (Ke *et al.* 2015) have been characterized with synthetic peptide. Synthetic hepcidin exhibited wide spectrum activity against Gram-positive and Gram-negative bacteria, fungi, virus and neoplastic cells.

Hepcidin is a moderately cationic and hydrophobic peptide with variety of physiological functions. Apart from direct immunological functions, it also possesses iron regulatory functions (Franchini *et al.* 2010). Upregulation of hepcidin transcripts with various other physiological stress like iron overload (Pereiro *et al.* 2012), hypoxia etc. could be observed (Attia *et al.* 2015). More than 70 hepcidin isoforms have been identified from various fishes (Masso-silva and Diamond 2014). However, they differ drastically in terms of amino acid composition and strain selectivity of microbes. There is marked difference in antimicrobial potential of isoforms within a species (Yang *et al.* 2011; Li *et al.* 2014). Therefore functional characterization of each hepcidin is an essential need to elucidate its biological role in innate immunity of fish. Present chapter deals with molecular and functional characterization of a synthetic hepcidin, Hepc-CB1 identified from deep sea fish Spiny-jaw Green-eye, *Chlorophthalmus bicornis*. Antimicrobial, anticancer and cytotoxic properties of the peptide are analyzed in this chapter.

6.2 Materials and Methods

6.2.1 Design and synthesis of target peptide

The mature bio-active peptide region of preprohepcidin, Hepc-CB1 (Fig. 6.1) identified from the deep sea fish *C. bicornis* was selected for the synthesis and functional characterization. Hepc-CB1 is a small cationic, β-sheet peptide with a predicted molecular weight of 2.53 kDa and hydrophobic index of 54% (Refer chapter 4, section 4.3.1.2 for more details). The peptide was synthesized at VCPBIO Limited, Shenzhen, China by Solid phase procedure of Fmoc chemistry from its C-terminus to N-terminus. The peptide was supplied as lyophilized powder and stored at -20 °C until use.

MKTFSVAVAVVLTFICLQQSSAVPVTEGEDPEVPMVDVYEEVPVES
WKMPYNNRLKRSAAGCKFCCGCCPDMNGCGVCCRF

Fig. 6.1: The 81 aa preprohepcidin identified from the fish *Zanclus cornutus*. Mature bio-active hepcidin (Hepc-CB1) is bold underlined

6.2.2 Determination of purity of the synthesized peptide

Purity of synthesized peptide, Hepc-CB1 was confirmed by Reverse phase HPLC (at VCPBIO, China) using Inertsil ODS-SP C18 column (4.6 mm x 250 mm). For purification, the sample was dissolved in 0.1% trifluoroacetic acid and 10 µl was injected into the column. Bound peptides were eluted by step gradient with solution A and B with a flow rate of 1 ml/min. Trifluoroacetic acid in acetonitrile (1ml in 1000 ml) and acidified water (0.1% trifluoroacetic in 100% H_2O) were used as solvent A and B respectively. Initially a gradient of 21% solvent A and 79% solvent B was

maintained for 0.01 min, followed by a gradient of 46% solvent A and 54% solvent B for 25 min and finally a flow rate of 100% solvent A for 5 min.

6.2.3 Determination of mass of the synthetic peptide

Mass of the synthetic peptide was determined at VCPBIO Ltd, China, by MALDI-TOF mass spectrometry using Thermo Finnigan LCQ Duo Mass Spectrometer. The analysis was performed using Xcaliber software with an electrospray source. For the analysis, the sample was dissolved in 50% (v/v) acetonitrile and 50% of 0.1% trifluoroacetic acid (v/v) and analysed by Electrospray Ionization (ESI) method. The spectra were obtained in the continuous acquisition mode, scanning from m/z 400 to 2000 at a scan time of 5 s.

6.2.4 Functional characterisation of synthetic Hepc-CB1

6.2.4.1 Antibacterial assay

6.2.4.1.1 Microbial strains used for the study

Antimicrobial activity of the synthetic Hepc-CB1 was tested against both Gram-positive and Gram-negative bacteria. *Bacillus cereus* (MCCB 101) and *Staphylococcus aureus* (MTCC 3061) were the Gram-positive bacteria used for the analysis. *Edwardsiella tarda* (MTCC 2400), *Pseudomonas aeruginosa* (MCCB 119), *Aeromonas hydrophila* (MCCB 113), *Vibrio cholerae* (MCCB 129) and *Vibrio parahaemolyticus* (MCCB 133) were the Gram-negative bacteria used for analysis.

6.2.4.1.2 Preparation of bacterial cell suspension

For the antimicrobial assay bacterial cell suspension was prepared in 50 mM HEPES buffer as described in Chapter 5 section 5.2.19.2.

6.2.4.1.3 Liquid growth inhibition assay

Antimicrobial activity of the synthetic Hepc-CB1was performed in a 96 well microtiter plates using Liquid growth inhibition assay described by Huang *et al.* (2006) with modifications. For details refer chapter 5 section 5.2.19.3. MIC was calculated against tested microorganisms using the software Priprobit as described in chapter 5 section 5.2.19.3.

6.2.4.2 Anticancer assay

Anticancer property of the synthetic hepcidin, Hepc-CB1was tested against 60 human tumour cell lines. The process was done as a part of *In Vitro* Cell Line Screening Project (IVCLSP) under developmental therapeutic programme of National Cancer Institute (NCI), Maryland, US. The screen was implemented in full-fledged form in 1990s to identify novel natural or synthetic compounds with potent antitumor activity that could develop into a promising anticancer agent. Sixty different human tumour cell lines were used for the screening. They represent leukaemia, melanoma, cancers of lung, colon, central nervous system, prostrate, ovary, renal and breast cancer cell lines. The details of cell lines used for the screening are given in Figures 6.10 - 6.18.

In vitro cell line screening is a multistep process. A virtual screening of the target peptide using computer software is the primary step and only those compounds which are showing satisfactory results in the virtual screening, will be allowed to pass into actual screening process. The actual screening/*in vitro* screening is a two stage process. As a preliminary screening, single dose of the peptide (10 μM) was tested against 60 human tumour cell lines. In the second stage, the five different concentrations of

the peptide were evaluated against the same 60 cell lines. However the second stage screening is done only for compounds showing a pre-determined threshold level inhibition against the tested cell lines in the preliminary single dose screening.

As a pre-requirement of the preliminary virtual screening, structure of the peptide (Hepc-CB1) in 'Mol' (.mol) format was sent to NCI. Since the results of the virtual screening found promising (Result not given by NCI); single dose screening was done at NCI, according to their standard cell line screening procedures. Briefly, the human tumor cell lines were grown in RPMI 1640 medium containing 5% foetal bovine serum and 2 mM L-glutamine for the screening process. Cells were inoculated into 96 well microtiter plates in 100 µl at plating densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. Prior to the addition of experimental drug, Hepc-CB1, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h.

Before the addition of test drug, Hepc-CB1, two plates of each cell line were fixed *in situ* with TCA to get a representative measurement of the cell population of each cell line at the time of addition of test drug (Tz). The test sample Hepc-CB1 was solubilized in dimethyl sulfoxide and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to the required test concentration with complete medium containing gentamicin (50 µg/ml). Aliquots of 100 µl of diluted drug was added to appropriate microtiter wells prefilled with 100 µl of the medium, resulting in the required final drug concentration (10 µM).

Subsequently the plates were incubated for an additional 48 h at 37 °C, 5% CO_2, 95% air, and 100% relative humidity.

For adherent cells, the assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 minutes at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the methodology was the same except that the assay was terminated by fixing settled cells at the bottom of the wells by gentle addition of 50 µl of 80% TCA (final concentration, 16% TCA). Using the seven absorbance measurements [the percentage growth was calculated at each of the drug concentration level. Percentage growth inhibition was calculated as,

$$[(Ti-Tz)/(C-Tz)] \times 100 \text{ for concentrations for which } Ti>/=Tz$$

Where 'Tz' is time zero, 'C' is control growth and Ti is the test growth in the presence of drug.

6.2.4.3 Cytotoxicity assay/(XTT) assay with NCI-H460 cells

Cytotoxicity of the synthetic Hepc-CB1 was tested against NCI-H460 cells by using XTT (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-

Tetrazolium-5-Carboxanilide) assay. Briefly, for the analysis, cells were inoculated at a density of 1×10^6 NCI-H460 cells into each well of a 96 well tissue culture plate containing minimal essential medium (MEM). The plates were then incubated for 12 h at 37 °C. Washed with phosphate buffered saline (PBS) and then the medium was exchanged with MEM containing a series of two fold dilutions of synthetic Hepc-CB1 ranging from 0.39 to 50 µM. Analysis was performed with positive and negative controls. Commercially available AMP, Melittin and blank medium without peptides were used as the positive and negative controls respectively. After incubation for 24 h at 37 °C, the cells were observed for morphological changes under Inverted phase contrast microscope (Leica, Germany). Fifty microliter of XTT reagent (5 mg/ml) was then added to each well and was incubated for 4 h at 37 °C. Formazan crystals were dissolved by the addition of 20 µl DMSO solution. The absorbance of each well was determined using the Microplate reader (Tecan Microplate Reader) at 450 nm. Cell viability of NCI-H460 cells was calculated as a percentage of viable cells in the peptide treated group versus control group.

6.3 Results

6.3.1 Determination of mass and purity of synthetic Hepc-CB1

Synthetic Hepc-CB1 (12.5 mg) was supplied by VCPBIO Ltd, China in lyophilized powdered form. Molecular weight of Hepc-CB1 (2530.07 Da) was verified by ESI mass spectroscopy. The mass spectrum shows the mass to charge ratio (m/z) from 400 to 2000 of all the ionized molecules present in the sample. The most abundant ion in the spectrum could be

seen at a mass to charge ratio of 844.16 and is corresponds to Hepc-CB1 ionized to +3 (rounded off MW = 2530 + $3H^+$ = 2530.48) (Fig. 6.2). The mass to charge ratio is thus 2532.48/3 = 844.16. Purity of the synthetic peptide was assured by Reverse Phase HPLC. The compound eluted as a single peak with area 5579756 and height 349376 with 72.01% purity at the retention time 9.18 min (Fig. 6.3).

6.3.2 Antibacterial assay of synthetic Hepc–CB1

Antimicrobial activity of the synthetic peptide against both Gram-negative and Gram-positive bacteria was tested using Liquid Growth Inhibition assay. Hepcidin was found to be active against all the tested bacterial strains except Gram-positive *B. cereus*. The result was summarised and shown in Table 6.1. The peptide expressed considerable reduction in growth and multiplication of *S. aureus*. Approximately 90% (89.1) reduction in growth was obtained at 35.57 µM. But further growth reduction could not be observed even after increasing the sample concentration. Maximum growth inhibition at the highest tested concentration was 90.45%. *B. cereus* was found to be resistant to the peptide. Hepc-CB1 exhibited more than 95% reduction in growth against all the five tested Gram-negative bacteria. *V. cholerae* was found to be the most sensitive (MIC 71.15 µM) and *E. tarda* was the least sensitive (MIC 726.8 µM). Graphical representation of the activity spectrum is shown in Fig. 6.4-6.9.

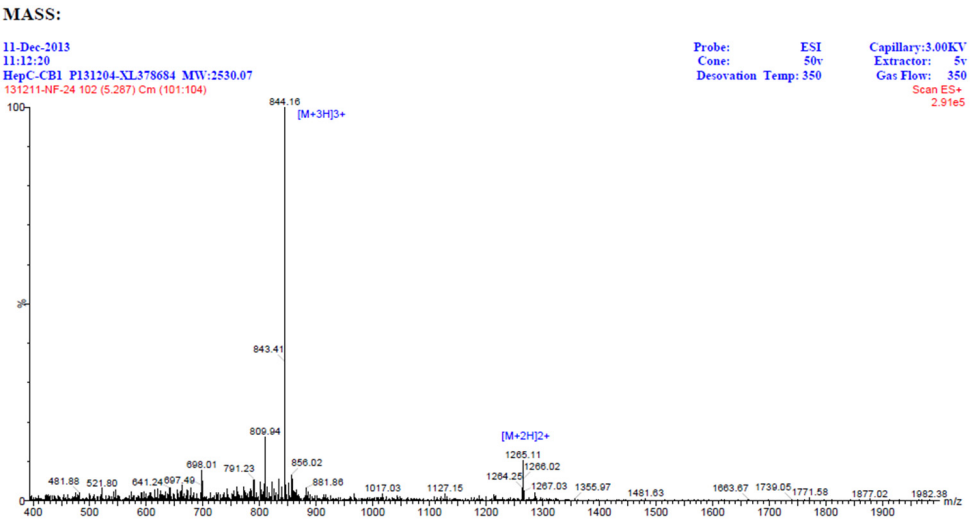


Fig. 6.2: ESI Mass Spectrum of Synthetic Hepc-CB1. Most abundant ion in spectrum is seen at m/z of 844.16 $[M+3H]3^{+}$ followed by 1265.1 [M+2H]2+

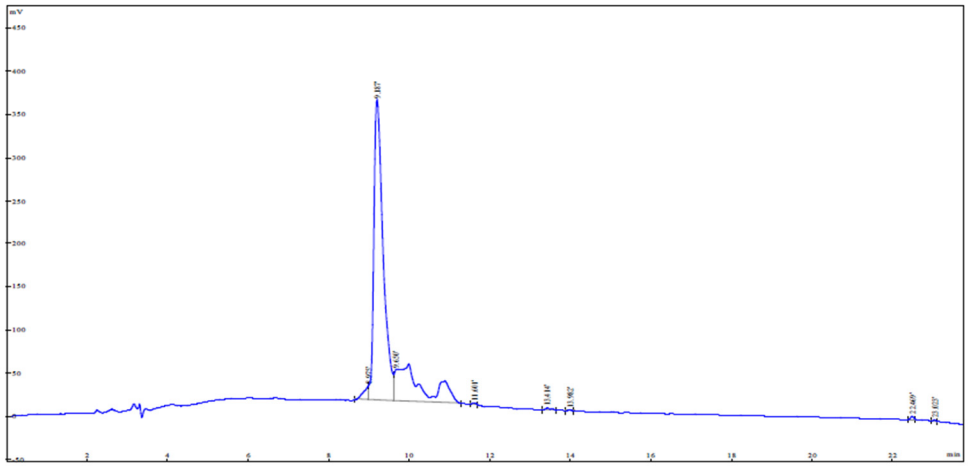


Fig. 6.3: HPLC chromatogram of synthetic Hepc-CB1. The compound eluted as a single peak with area 5579756 and height 349376 with 72.01 % purity at the retention time 9.18 min

Table 6.1: Antimicrobial activity of synthetic Hepc-CB1 against various Gram-positive and Gram-negative bacteria

Con. Molarity (µM)	*S. aureus*	*V. cholerae*	*E. tarda*	*P. aeruginosa*	*V. parahaemolyticus*	*A. hydrophila*
142.29	90.45	96.82	70.53	94.09	95.25	97.63
71.15	89.65	96.77	56.79	90.55	94.59	90.93
35.57	89.1	96.67	53.56	73..4	85.53	80.04
17.79	88.25	94.38	42.44	54.3	84.5	57.71
8.89	87.9	75.84	35.67	34.38	63.89	8.12
4.45	75.4	52.7	9.6	7.4	32.87	0
MIC in µM	**620**	**71.15**	**726.8**	**261.9**	**228.16**	**142.29**

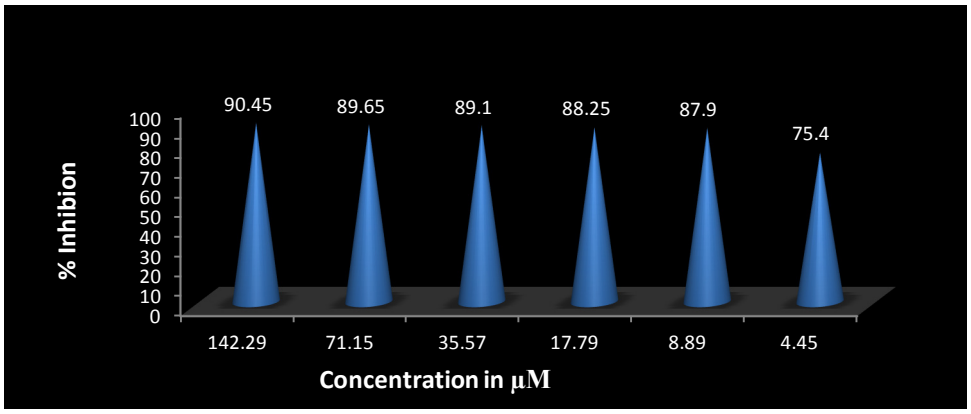


Fig. 6.4: Antimicrobial activity of Synthetic Hepc-CB1 against *S. aureus*

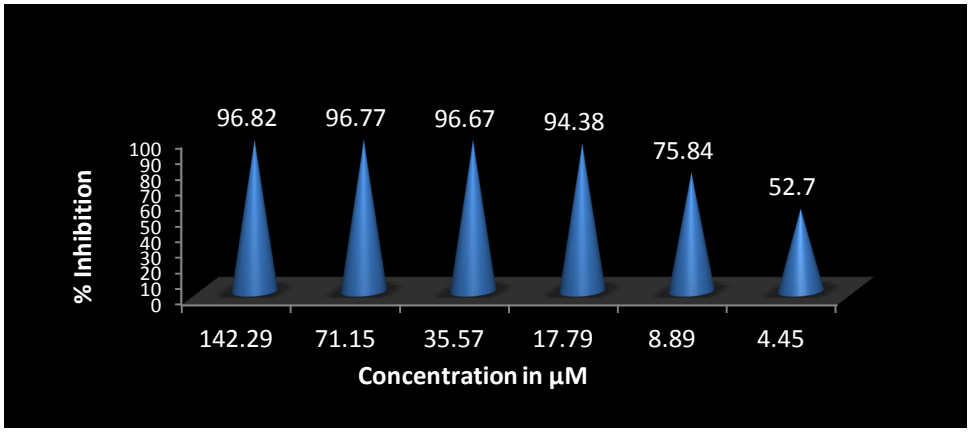


Fig. 6.5: Antimicrobial activity of Synthetic Hepc-CB1 against *V. cholerae*

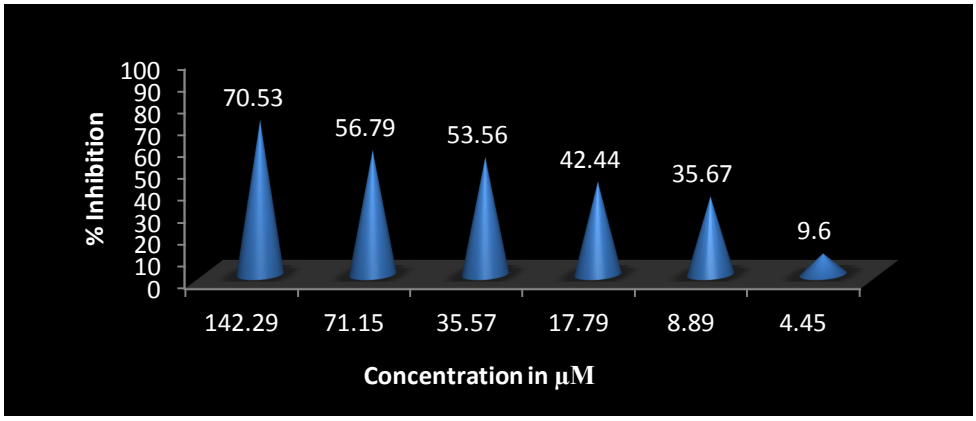


Fig. 6.6: Antimicrobial activity of Synthetic Hepc-CB1 against *E. tarda*

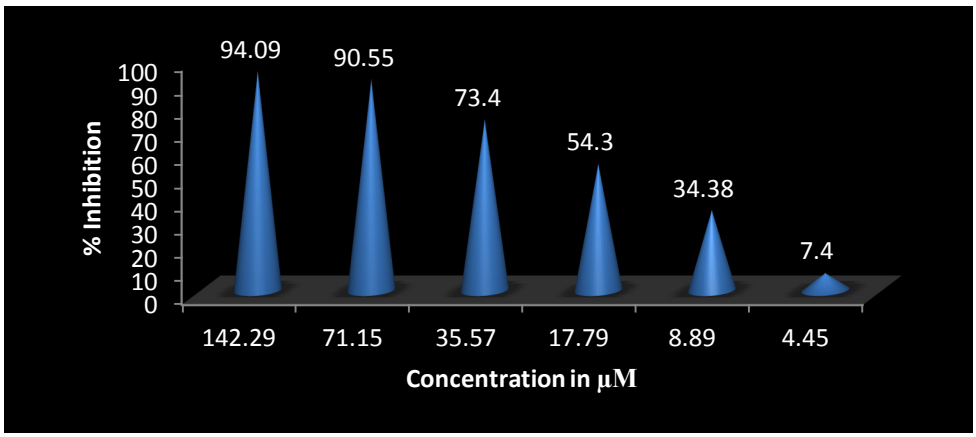


Fig. 6.7: Antimicrobial activity of Synthetic Hepc-CB1 against *P. aeruginosa*

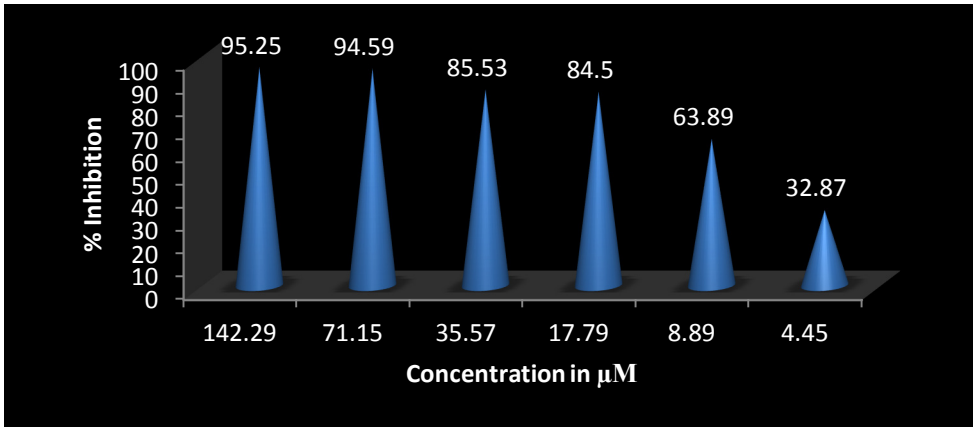


Fig. 6.8: Antimicrobial activity of Synthetic Hepc-CB1 against *V. parahaemolyticus*

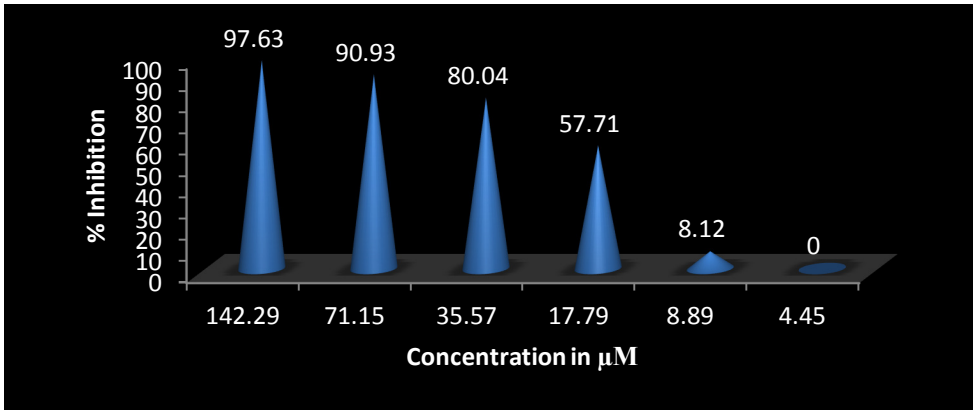
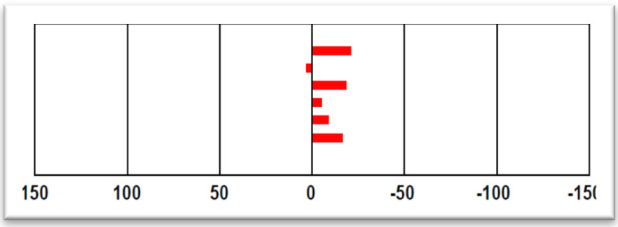


Fig. 6.9: Antimicrobial activity of Synthetic Hepc-CB1 against *A. hydrophila*

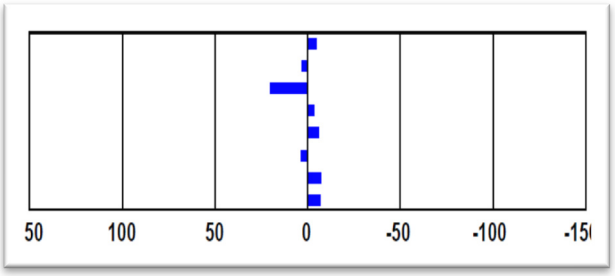
6.3.3 Anticancer assay of Hepc-CB1

Anticancer activity of Hepc-CB1 was tested under *In Vitro* Cell Line Screening Project **(IVCLSP)** of Development Therapeutics Program NCI-60 (National Cancer Centre, US). No significant anticancer property was exhibited by the peptide against any of the tested cell lines except Leukaemia cell lines. Only 10-12% growth reduction could be obtained against Leukaemia cell lines. Growth inhibition of the synthetic hepcidin against the tested cancer lines are summarised in Fig. 6.10 to 6.18.



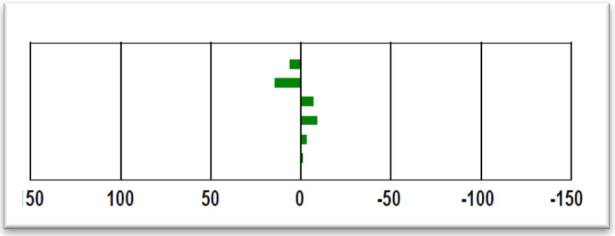
CCRF-CEM	88.41
HL-60(TB)	111.94
K-562	90.88
MOLT-4	104.04
RPMI-8226	100.40
SR	93.06

Fig. 6.10: Activity of synthetic Hepc-CB1 against Leukasemia cell lines



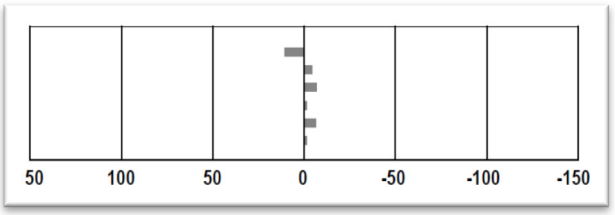
A549/ATCC	104.55
HOP-62	111.97
HOP-92	128.98
NCI-H226	105.70
NCI-H23	103.21
NCI-H322M	112.57
NCI-H460	102.14
NCI-H522	102.71

Fig. 6.11: Activity of synthetic Hepc-CB1 against Non-Small Cell Lung Cancer cell lines



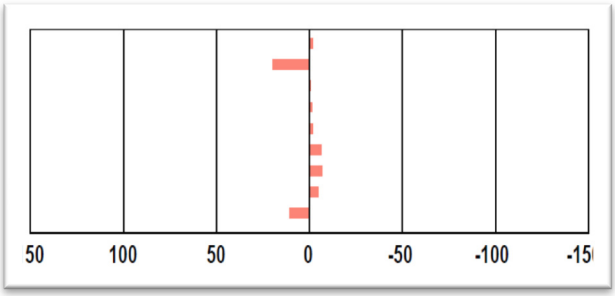
COLO 205	115.06
HCC-2998	123.50
HCT-116	102.38
HCT-15	100.47
HT29	106.30
KM12	108.24
SW-620	109.18

Fig. 6.12: Activity of synthetic Hepc-CB1 against Colon Cancer cell lines



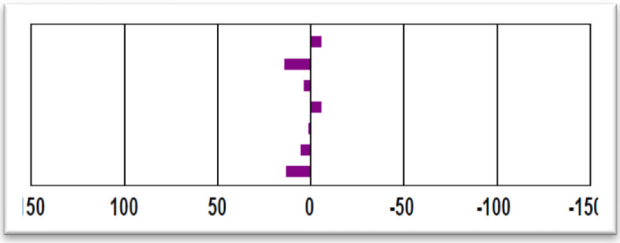
SF-268	119.67
SF-295	105.15
SF-539	102.60
SNB-19	108.11
SNB-75	102.84
U251	107.94

Fig. 6.13: Activity of synthetic Hepc-CB1 against CNS cancer cell lines



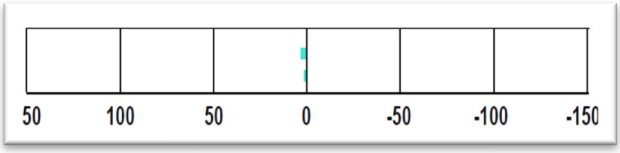
LOX IMVI	107.38
MALME-3M	128.89
M14	108.99
MDA-MB- 435	107.88
SK-MEL-2	107.72
SK-MEL-28	102.82
SK-MEL-5	102.54
UACC-257	104.46
UACC-62	119.76

Fig. 6.14: Activity of synthetic Hepc-CB1 against CNS cancer cell lines



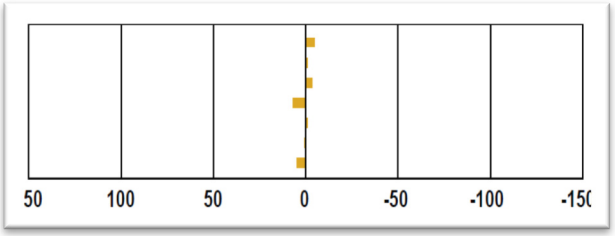
IGROV1	103.84
OVCAR-3	123.02
OVCAR-4	112.73
OVCAR-5	103.89
OVCAR-8	110.20
NCI/ADR-RES	114.37
SK-OV-3	122.28

Fig. 6.15: Result of anticancer assay of synthetic Hepc-CB1 against Ovarian cancer cell lines



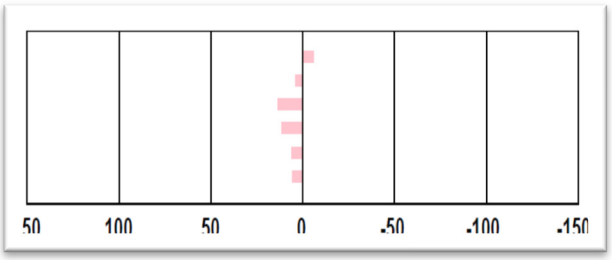
PC-3	112.09
DU-145	110.27

Fig. 6.16: Activity of synthetic Hepc-CB1 against Prostrate cancer cell lines



786-0	104.79
ACHN	108.42
CAKI-1	105.77
RXF 393	115.77
SN12C	108.56
TK-10	109.61
UO-31	114.00

Fig. 6.17: Activity of synthetic Hepc-CB1 against Renal cancer cell lines



MCF7	103.61
MDA-MB-231	113.01
HS 578T	122.37
BT-549	120.55
T-47D	114.90
MDA-MB-468	114.67

Fig. 6.18: Activity of synthetic Hepc-CB1 against Breast cancer cell lines

6.3.4 Cytotoxicity assay of Hepc-CB1

Cytotoxicity of Hepc-CB1 was tested against Human non-small lung carcinoma (NCI-H460) Cell line using XTT assay. Cytotoxicity was less than 10% at the highest tested concentration and it remained more or less same over the various concentrations tested (6-9%). The peptide was twice less cytotoxic than the positive control (magainin) used. Magainin exhibited approximately 20% toxicity against the tested cell lines. Result of cytotoxicity assay is shown in Fig. 6.19.

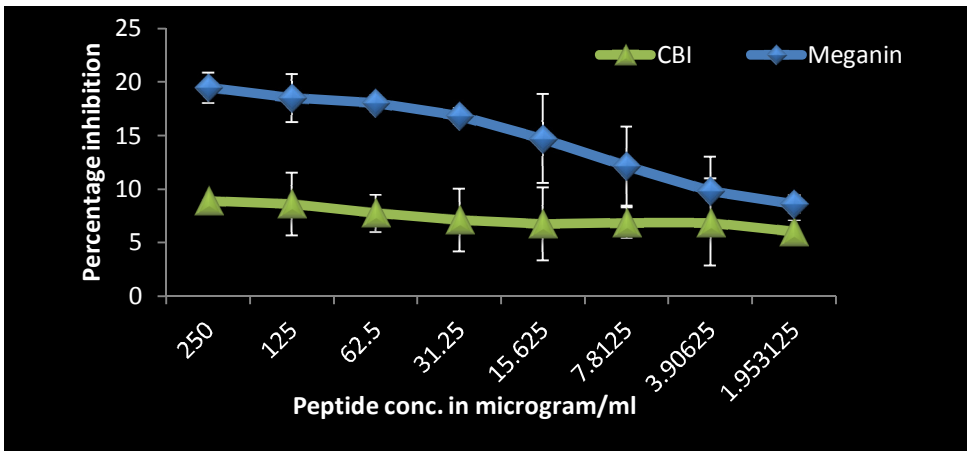


Fig. 6.19: Activity of synthetic Hepc-CB1 against NCI-H460 cells

6.4 Discussion

Hepc-CB1 is cationic (+3), low molecular weight, moderately hydrophobic (54%) peptide with 8 cysteines identified from the deep-sea fish Spiny-jaw Green-eye (*Chlorophthalmus bicornis*). The peptide was synthesized by solid-phase technology with 72.01% purity using Fmoc chemistry. Antimicrobial property of the peptide was assessed with two strains of Gram-positive and five strains of Gram-negative bacteria.

Hepc-CB1 exhibited strong microbicidal activity against all the five Gram-negative bacteria tested and *S. aureus*; while it was found insensitive towards the Gram-positive bacteria *B. cereus*. Most of the synthetic hepcidins exhibited activity against various strains of *S. aureus* and the activity could be detected down to 1.5 μM concentration (Wang *et al.* 2009; Cai *et al.* 2012; Ke *et al.* 2015). Similarly, Hepc-CB1 also exhibited 75% inhibition of the tested *S. aureus* strain at a concentration of 4.45 μM. Nearly 88% reduction of *S. aureus* could be observed at 17.79 μM concentration and no further reduction in growth could be observed even after increasing the concentration of the peptide. According to previous reports *B. cereus* is relatively resistant to hepcidin (Yang *et al.* 2011). However the hepcidin isoform identified from the fish, Large yellow croaker, *Pseudosciaena crocea* was found to inhibit *Bacillus* sp. (Wang *et al.* 2009). Synthetic hepcidin in general displayed fairly good antimicrobial property against Gram-negative bacteria. It showed microbicidal activity against *Escherichia coli, Aeromonas hydrophila, V. harveyi, V. anguillarum, V. parahaemolyticus, V. alginoltyticus, V. damsela, V. vulnificus, Photobacterium damselae, Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae, Shigella sonneri, S. flexneri, Yersinia enterocolitica, Listeria monocytogenes* and *Piscirickettisia salmonis* (Lauth *et al.* 2001, 2005; Huang *et al.* 2007; Wang *et al.* 2009; Yang *et al.* 2011; Cuesta *et al.* 2011; Cai *et al.* 2012; Álvarez *et al.* 2014; Zhang *et al.* 2014; Li *et al.* 2014; Ke *et al.* 2015). Consistently all the five tested Gram-negative bacteria exhibited fairly well sensitivity to the tested peptide. More than 90% reduction could be observed at the maximum tested concentration for all the tested bacteria except *E. tarda* which was found to

be the most resistant bacteria in the present study. Sm Hep2P characterized from turbot (*Scophthalmus maximus*) was the only reported hepcidin that exhibited bactericidal activity against *E. tarda* (Zhang *et al.* 2014). At the same time, another hepcidin isoform (Sm Hep1P) identified from the same fish did not show any activity against the same strain of *E. tarda* tested under the same test conditions. All these observations support the assumption that different hepcidin isoforms behave differently against the same pathogens (Cai *et al.* 2012). *V. cholerae* was found to be the most sensitive bacteria with 75.84% growth inhibition at the maximum tested concentration (142.29 µM) and there are no previous reports of antimicrobial activity of synthetic hepcidin against *V. cholerae*.

In literature, the average range of MIC reported for hepcidin against Gram-negative bacteria was 1.5 to 60 µM. In the present study, more than 90% reduction in growth has been observed in all tested bacteria at 71.15 µM concentration except *E. tarda*. The test peptide used for the study had only 72.01% purity. Furthermore, it is very difficult to synthesize properly folded bioactive hepcidin due to the presence of disulphide bonds (Klüver *et al*. 2006; Wu *et al*. 2003). Low purity and improper structuring of synthetic hepcidin due to misfolding of disulphide bonds could have been the reason for low antimicrobial activity and elevated MICs of synthetic Hepc-CB1. Without or misfolded disulphide bonds in hepcidin could form an α-helical configuration, the reduced form of hepcidin; which is biologically less active than original configuration (Álvarez *et al.* 2014). For specific and stronger antimicrobial activity, proper disulphide bonding is essential (Álvarez *et al.* 2014; Lin *et al.* 2014). Though the recombinant hepcidin exhibited activity against only vibrios, compared to synthetic hepcidin, the

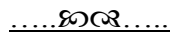
activity was stronger which is evident from the lower MIC values obtained (Please see chapter 5). The stronger activity exhibited by recombinant peptide could be due to the proper disulphide bonding.

Cancer remains a major cause of morbidity and mortality despite all recent advances in cancer immunology. Recent reports have portrayed antimicrobial peptides/anticancer peptides as a promising class of chemotherapeutic agents due to the selective toxicity to negatively charged cancer cells and relative non-toxicity to normal mammalian cells (Chen *et al.* 2014). Peptide mediated chemotherapy created a new wave in cancer therapy and it could be used in different ways. This include direct use of peptides (as angiogenesis inhibitors), tumor targeting agents that carry cytotoxic drugs and radionuclides (targeted chemotherapy and radiation therapy), hormones and vaccines (Thundimadathil 2012). Some fish AMPs (epinecidin, pardaxin, pleurocidin) including tilapia hepcidin (Th2-3) expressed potential anticancer activites to various human cancer cells (Chen *et al.* 2009b,a; Lin *et al.* 2009; Hsu *et al.* 2011a,b; Ting *et al.* 2014). Anticancer properties of Hepc-CB1 was also assayed against 60 human cancer cells as a part of *In Vitro* Cell Line Screening Project (IVCLSP) of Development Therapeutics Program NCI-60 (National Cancer Centre, US). Hepc-CB1 was qualified in structure based primary virtual screening for anticancer potential. In secondary *in vitro* screening, it exhibited 12, 10 and 7% reduction against the leukaemia cell lines ie, CCRF-CEM, K-562 and SR at 10 μM concentration. Those compounds having inhibitory activity greater than or equal to 50 only will be considered for further analysis as per NCI (USA) guidelines. The inhibition percentage of Hepc-CB1 on *in vitro* screening did not reach the prescribed 50% inhibition level. If

N-terminal acetylation, C-terminal amidation and folding through disulphide bridges could have been effected, antimicrobial and anticancer properties of the hepcidin would have been better.

Cytotoxicity of the synthetic hepcidin, Hepc-CB1 was tested with NCI-H460 cell lines using XTT assay. Cytotoxicity of hepcidins has not been reported till date. Cytotoxicity of Hepc-CB1 at the highest tested concentration was less than 10% and well below that of magainin. Similarly Hepc-CB1 is proved as a non-cytotoxic peptide with antimicrobial potential and therefore can be subjected to further investigations for developing into a drug.

Hepc-CB1 is a peptide with all the hallmarks of a classical antimicrobial peptide and hence has the potential to develop as a therapeutic agent. Recombinant expression in suitable vectors or even chemical synthesis with secondary modifications could be done to get active peptide. The peptide could also be used as templates to develop novel drugs. More investigations are required to establish the biological properties and functional role of the peptide in natural habitats.



Chapter 7

SUMMARY AND CONCLUSION

Antimicrobial peptides are evolutionarily conserved components of host innate immune defense of organisms spanning over the phylogenetic spectrum. Despite their diverse origin, amino acid composition and structure, AMPs have some biophysical features in common that are likely to be essential for biological activity like small size, net charge (generally positive) and amphipathic nature. Many of the AMPs have been known to possess wide spectrum antimicrobial properties besides immunomodulatory and chemotactic properties.

Bacterial resistance to conventional antibiotics is a serious socio-economic concern and global public health problem. More than 70% of bacteria are resistant to at least one of the antibiotics prescribed today and some of them are multiple drug resistant also. This led the scientific community to search for novel therapeutic agents, which can overcome bacterial resistance. Here comes the significance of AMPs, which are natural antibiotics produced by almost all organisms that can neutralize a wide range of microbes without developing microbial resistance. AMPs are promising candidates in the field of pharmacology and medicine as therapeutic agents that could overcome bacterial resistance.

The present study was focused on molecular and functional characterization of novel antimicrobial peptides in marine fishes. Fishes belonged to different marine habitats were screened for the presence of novel Antimicrobial peptides through a gene based approach. Total RNA was extracted from fish tissues; cDNA was synthesized and amplification of the AMP genes was carried out with gene specific primers. Amplicons were sequenced and identities of the AMP genes were confirmed through BLAST analysis at NCBI. Molecular and structural characterization of the peptides was carried out with various bioinformatics tools. Functional characterization of two novel hepcidin isoforms was carried out with recombinant and synthetic peptides by employing antimicrobial/ anticancer/cytotoxicity assays.

Salient findings

- In the present study marine fishes belonging to different ecological niches of the marine ecosystem were screened using specific primers for the presence of important antimicrobial peptide families such as piscidin, hepcidin, histone-derived peptides as well as cathelicidins. Altogether 5 AMPs, belonging to the families piscidins, hepcidins and histone-derived peptides could be identified and characterized.
- Epinecidin, a piscidin family peptide identified from the genus *Epinephelus coioides,* collected from Cochin estuary, showed 100% similarity in amino acid sequence to Epinecidin 1 of *E. coioides* and Piscidin 2 of *E. malabaricus* of the South Chinese Sea.

- Though differences in nucleotide sequences were observed, the AMP epinecidin was found to be conserved within the genus irrespective of the geographical variation and subsequent selective pressures exerted by the highly dynamic external environment.
- Piscidins identified from the genus *Epinephelus* could be grouped into four main types based on the amino acid sequence difference in the N- and C-terminus of the mature peptide region.
- A 245 bp amplicon coding for 52 aa termed as teleostin could be amplified from the blood cells of Bengal tongue-sole, *C. semifasciatus* and Catfish, *T. jella*.
- The 52 aa Teleostin is an α-helical peptide with a predicted molecular weight of 5.527 kDa, a theoretical isoelectric point of 12.01, a net positive charge of +12, total hydrophobic index of 32% and a protein binding potential of 2.38 kcal/mol.
- An 87 aa preprohepcidin with 24 aa signal peptide, 39 aa prodomain and a 24 aa mature peptide could be identified and characterized from the fish *Chlorophthalmus bicornis*. The 24 aa mature peptide was termed as Hepc-CB1.
- Hepc-CB1 was found to possess a molecular weight of 2.53 kDa, a net positive charge (+3) and capacity to form β-hairpin-like structure configured by 8 conserved cysteines.

- Phylogenetic analysis categorized Hepc-CB1 to HAMP2-like group. This is the first report of a hepcidin isoform under the group HAMP2 from a non-acanthopterygian deep-sea fish.
- A 81 aa preprohepcidin possessing hydrophobic aa rich 22 mer signal peptide, a highly variable proregion of 35 aa and 24 aa bioactive mature peptide with 8 conserved cysteine residues was obtained from the coral reef fish Moorish idol, *Z. cornutus*. The 24 aa mature peptide is termed as ZC-Hepc1.
- Zc-Hepc1 is a β-hairpin-peptide, possessing a theoretical isoelectric point of 7.46, a predicted molecular weight of 2.43 kDa and a net positive charge of +1. Phylogenetic analysis grouped *Z. cornutus* hepcidin to HAMP2 group hepcidins. This is the first report of an AMP from the coral fish *Z. cornutus*.
- Recombinant expression of Zc-Hepc1 was successfully carried out in Rosetta-gami^{TM} B (DE3) pLysS expression host system using pET32a+ expression vector system.
- Antimicrobial activity of the recombinant peptide was tested against both Gram-positive and Gram-negative bacteria using liquid growth inhibition assay. Recombinant Zc-Hepc1 showed significant activity against *V. cholerae* (MIC=30.36 μM) and *V. parahaemolyticus* (MIC=57.15 μM).
- Mature peptide region of the novel Hepcidin isoform identified from *Chlorophthalmus bicornis*, Hepc-CB1 was synthesized by solid phase peptide synthesis at VCPBIO Ltd., China. Functional

characterization of the peptide was performed by antimicrobial, anti-cancer and cytotoxicity assays.

- Synthetic Hepc-CB1 was found to be active against both Gram-positive bacteria, *S. aureus* and all the five tested Gram-negative bacteria such as *Vibrio parahaemolyticus*, *V. cholerae*, *P. aeruginosa, E. tarda* and *A. hydrophila*.
- Synthetic Hepc-CB1 was also tested for anticancer activity at a concentration of 10 μM against 60 human cancer cell lines categorized under Colon Cancer, Non-Small Cell Lung Cancer, CNS Cancer, Renal Cancer, Melanoma, Ovarian Cancer, Breast Cancer, Prostate Cancer and Leukaemia. A growth inhibition of 10-12% against Leukaemia cell lines alone could be observed. Cytotoxicity assay of Hepc-CB1 carried out against Human epithelial type 2 (NCI-H460) cell lines proved the synthetic peptide to be non-cytotoxic.
- All the nucleotide sequences and the deduced amino acid sequences characterized in the present study were submitted to GenBank under the following accession numbers

  a) Epinecidin from *Epinephelus coioides* – ID: KX090373
  b) Teleostin from *Cynoglossus semifascitus* -ID: HQ720152
  c) Teleostin from *Tachysurus jella* - ID: HQ720153
  d) Hepcidin from *Chlorophthalmus bicornis* - ID: JX163299
  e) Hepcidin from *Zanclus cornutus* - ID: JX163300

Fishes are the transition forms between organisms which relies intensely upon innate immunity and adaptive immunity. Although fishes

are able to develop adaptive immune responses, they mainly depend on non-specific immune parameters. AMPs are an inevitable element of primary defense of fish. Marine fishes are relatively unexploited resource in terms of AMPs. Present study was carried out to identify novel AMPs from fishes. Fishes belonging to various marine habitats were screened for the presence of novel AMPs. As an outcome of the study an epinecidin, two histone-derived peptides (teleostin) and two novel hepcidin isoforms (Hepc-CB1 and Zc-Hepc1) were identified and characterized. Phylogenetic analysis and evolutionary significance of AMPs were also analyzed and substantiated through the study. Besides, the functional characterization of the hepcidin isoforms were also carried out with both recombinant and synthetic peptide in terms of antimicrobial, anticancer, as well as cytotoxic assays. Both recombinant and synthetic hepcidins have the potential to be developed into novel therapeutic agents. Amino acid modifications and terminal modifications would result in improved antimicrobial potential and stability of the peptide *in vitro* and *in vivo*. Post translational modifications and folding are essential for hepcidin to be an active molecule. Hepcidin would definitely be a promising candidate with potential to develop into a novel chemotherapeutic agent. Non-cytotoxicity of the peptide is also an added advantage for its applications. Further research is highly warranted for the application of AMPs as novel therapeutic agents especially in case of drug resistance.

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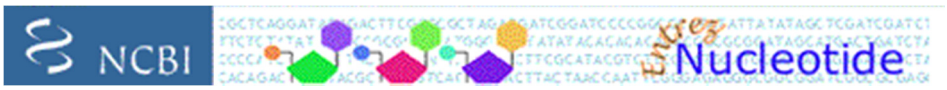
Appendix

Table 1 Details of fish Samples collected for the study

Sl. No	Scientific Name	Common Name	Sampling Location
1	*Gnathanodon speciosus*	Golden travelly	Off Trivandrum
2	*Odonus niger*	Trigger fish	Off Trivandrum
3	*Antennarius coccineus*	Angler fish	Off Trivandrum
4	*Megalaspis cordyla*	Horse mackeral	Neendakara, Kollam
5	*Nemipterus mesoprion*	Thread fin bream	Neendakara, Kollam
6	*Gymnothorax favagineus*	Muray eel	Sakhthikulangara, Kollam
7	*Platax teira*	Tiera batfish	Off Kollam
8	*Sillago sihama*	Indian whiting	Alleppy, Kerala
9	*Epinephelus coioides*	Grouper	Cochin Estuary
10	*Terapon jarbua*	Crescent Grunter	Cochin Estuary
11	*Lutjanus russelli*	Russell's Snapper	Cochin Estuary
12	*Collichthyes dussumeri*	Frog fish	Cochin Estuary
13	*Etroplus suratensis*	Pearl Spot	Cochin Estuary
14	*Scatophagus argus*	Spotted Scat	Cochin Estuary
15	*Periophthalmus minutus*	Mud skipper	Cochin Estuary
16	*Trypauchen vagina*	Blind Gopi	Kalamukku Harbour, Kochi
17	*Tachysurus/Arius jella*	Black-fin sea catfish	Kalamukku Harbour, Kochi
18	*Cyanoglossus semifasciatus*	Bengal tongue sole	Kalamukku Harbour, Kochi
19	*Menae macculata*	Moon fish	Kalamukku Harbour, Kochi
20	*Secutor insidiator*	Silver belly	Kalamukku Harbour, Kochi
21	*Gaza minuta*	Silver belly	Kalamukku Harbour, Kochi
22	*Leognathus equulus*	Silver belly	Kalamukku Harbour, Kochi
23	*Parambassis dayi*	Nandan	Vypeen, Kochi
24	*Gerrous filamentosus*	Whip tail Silver Biddy	Fort Kochi
25	*Mugil cephalus*	Mullet	Fort Kochi
26	*Alectis indicus*	Indian thread fish	Fort Kochi
27	*Tetradon biocellatus*	Puffer fish	Fort Kochi
28	*Liza parsia*	Mullet	Fort Kochi
29	*Zanclus cornutus*	Moorish Idol	Off Kochi
30	*Sardinella longiceps*	Oil sardine	Chavakkad, Thrissur, Kerala
31	*Rasrelliger Kanagurta*	Indian Mackeral	Chavakkad, Thrissur, Kerala
32	*Stolephorus indicus*	Anchovy	Chavakkad, Thrissur, Kerala
33	*Bregmaceros*	Unicorn cod	Off Calicut

34	*Corephynia*	Dolphin Fish	Off Chennai
35	*Priacanthus macracanthus*	Red Big Eye	Off Chennai
36	*Harpodon squamosus*	Bombay duck	Off Karaikkal
37	*Nezumia brevirostris*	Rat tails	Off Karaikkal
38	*Lamprogrammus niger*	Cusk eels	Off Karaikkal
39	*Neoscopilus microchir*	Short fin lantern fish	Off Karaikkal
40	*Bathyclupea hoskynii*	Deep Sea Scaly Fins	Off Cuddalore
41	*Bathygadus furvescens*	Black fin Rat tails	Off Cuddalore
42	*Lamprogrammus fragilis*	Pike Congers	Off Cuddalore
43	*Lophioides mutilus*	Smooth Angler	Off Cuddalore
44	*Chlorophthalmus bicornis*	Spny jaw green eye	Andamaan sea
45	*Chauliodus sloani*	Barbeled Dragon fish	Andaman Sea
46	*Neobythites steatiticus*	Cusk eels	Andaman Sea
47	*Coloconger raniceps*	Frog head eel	Andaman Sea
48	*Chaunax pictus*	Pink Frog Mouth	Andaman Sea
49	*Dicrolene introniger*	Cusk eel	Andaman Sea
50	*Gavialiceps taeiniola*	Pike Congers	Andaman Sea
51	*Hypopleuron caninum*	Whip tail cusk eel	Andaman Sea
52	*Satyrichthys laticeps*	Armoured sea Robins	Andaman Sea
53	*Scalicus orientalis*	Armoured sea Robins	Andaman Sea
54	*Chascanopsetta lugubris*	Pelican flounder	Andaman Sea
55	*Bembrops platyrhyncus*	Natal Duckbill	Andaman Sea
56	*Pycnocraspedum squamipinne*	Cusk eel	Andaman Sea
57	*Monomitopus nigripinnis*	Cusk eel	Andaman Sea
58	*Glyptophidium oceanium*	Cusk eels	Andaman Sea
59	*Brotula multibarbata*	Goats beard brotula	Andaman Sea
60	*Bassozetus robustus*	Robust Ass fish	Andaman Sea
61	*Alepocephalus blanfordii*	Slick-head	Andaman Sea
62	*Alepocephalus bicolor*	Slick-head	Andaman Sea
63	*Synagrops japonicus*	Black mouth split-fin	Andaman Sea
64	*Synagrops adeni*	Aden split-fin	Andaman Sea
65	*Ostracoberyx dorygenys*	Shell-skin alfonsinos	Andaman Sea
66	*Setarches guentheri*	Channelled rockfish	Andaman Sea

GenBank Submissions



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