Antimicrobial Peptides: An Alternative to Antibiotics for Environment-Friendly Hill Aquaculture

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Abstract

Antimicrobial peptides (AMPs) are emerging as promising alternative to antibiotics, especially due to their activity against drug-resistant pathogenic strains. A vast number of AMPs have been characterized and purified from various natural sources, ranging from bacteria, fungus, plants, to higher vertebrates including mammals. Many of them have also been produced using either chemical synthesis or the recombinant technology. Both synthesized and expressed AMPs showed significant inhibitory activity against a number of economically significant pathogens. Majority of AMPs kill pathogens by disruption of cell membranes. Hence, chances of developing resistance by the microbes are less. Fish are reservoirs of these peptides, and they express majority of AMPs. However, application of AMPs in field condition is not at advanced stage, which may be due to issues like cost of production, stability, and toxicity to host cells. This can be addressed through artificial designing of short peptides to reduce the manufacturing cost and to enhance stability. AMPs not only kill microbes directly but also help in immunomodulation and may be highly useful for fish, which mainly depends on its innate immune system to fight against pathogens.

Keywords

Antimicrobial peptides · Classification · Mechanism of action · Fish · Artificial AMP · Production of AMP

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17.1 Introduction

Natural defense systems are vital for survival of organisms in varying conditions and environments. In vertebrates, this system is composed of two subdivisions, the innate immune system and the adaptive immune system. The innate immune system acts quickly and non-specifically providing the first line of defense against invading pathogens. It is not specific to a particular antigen and has no immunologic memory. On the other hand, adaptive immunity is antigen specific and has memory, enabling the host to produce effective immune response on later exposure to the antigen (Marshall et al. 2018). In fish, innate immunity is the central mechanism of defense as their adaptive immune system has some constraints such as limited repertoire of antibodies, affinity maturation and memory, and slow proliferation of lymphocytes (Magnadóttir 2006).

Innate immunity acts through defense mechanisms, physical barriers such as the skin which prevents the entry of pathogens, cellular components (e.g., macrophages), and humoral responses (e.g., antimicrobial peptides), which may produce direct bactericidal effects (Magnadóttir 2006). Antimicrobial peptides (AMPs) are gene-encoded, ribosomally synthesized, host defense peptides carrying a net positive (cationic) and has molecular weight less than 10 kDa (de Zoysa et al. 2015; Raju et al. 2020). AMPs are mainly encountered at the portal of pathogen entry that encompass circulating myeloid cells and skin and mucosal and epithelial surfaces where they are usually stored in secretory granules, destined for extracellular secretion (Adem Bahar and Ren 2013). The main tissues or organs of fish that are at the high-risk targets of pathogens are epithelial cells of the skin, gills, gastrointestinal tract, gut, and respiratory organs. Hence, AMPs are typically localized in these tissues (Scocchi et al. 2016). AMPs are synthesized as pre-proproteins that include a signal sequence which undergoes hydrolytic degradation to form the active molecules (Raju et al. 2020). They are conserved effector molecules that play a key role in the first line of defense against invading pathogens prior to the activation of adaptive immunity. Although these peptides are enormously diverse in terms of their biosynthesis, sequences, and structures, they all share certain structural characteristic features like small size (<100 amino acids), cationic nature at physiological pH, amphipathicity, broad-spectrum antimicrobial activity, and similar mode of action (Sathyan et al. 2012; Chaithanya et al. 2013). The cationic nature of AMPs helps them in initial binding with the anionic bacterial surface, and their amphipathic nature enables them to ravage the bacterial membrane structure, ultimately killing the bacteria (Brogden 2005). In most organisms, AMPs are multifunctional molecules that are involved not only in attacking the pathogen but also in other important biological functions like immune modulation, wound healing, and cancer cell growth inhibition and/or as signaling molecules, for example, hepcidins, besides acting as antimicrobial, are also involved in iron regulations (Shi and Camus 2006; Tincu and Taylor 2004). The common functional characteristic of most AMPs is their induction, following the exposure of host to pathogen-associated molecular patterns (PAMPs) (Rončević et al. 2020). Their expression is either constitutive (within secretory cells) or upregulated during an infection, highlighting their role as

antimicrobial. The induction pathways for AMP synthesis have been conserved evolutionarily in almost all organisms (Hancock and Diamond 2000; Acosta et al. 2019).

Increase in antibiotic-resistant bacteria and re-emergence of various infectious diseases have stimulated the exploration of these evolutionarily ancient components of the defense system as new therapeutic candidates to replace the conventional antibiotics and as cancer therapeutics. AMPs show exceptional specificity against prokaryotes, with low toxicity toward eukaryotic cells. Further, their rapid mode of killing mechanism gives very narrow escape for microbes to develop resistance (Chen and Lu 2020; Deslouches et al. 2015). They are highly effective even at micromolar concentrations and often exhibit synergistic effects with conventional antibiotics (Hancock and Diamond 2000; Yan and Hancock 2001). When many antibiotic treatments result in sepsis due to release of endotoxins from dead bacterial cells, AMPs bind to those endotoxins, reducing the septic shock (Bao et al. 2006). They have broad-range activity against Gram-positive and Gram-negative bacteria, fungi, some parasites, and enveloped viruses and on cancer cells. AMPs are one of the most promising alternatives to antibiotics because they are effective even against multidrug-resistant pathogens (Rima et al. 2021). Many researchers are also with the opinion that AMPs could offer the best promising solution to fight against microbial infection in aquaculture with no harmful effect on the environment (Chaturvedi et al. 2020; Valero et al. 2020). Interestingly, many AMPs, including those originated from fish, are being discovered and studied. Fish are considered as potential sources of AMPs, owing to their innate immunity being the major defense system. Here, we discuss the naturally occurring AMPs derived from fish, artificial designing of AMPs, and mechanism of action and highlight the possibility of their large-scale production for therapeutic applications.

17.2 History of Antimicrobial Peptides

AMP was discovered in 1939, when an antimicrobial agent named gramicidin, isolated from a soil bacterium, *Bacillus brevis*, protected mice from pneumococcal infection (Dubos 1939). Hirsch (1956) reported the first AMP of animal origin, defensin, isolated from leukocytes of rabbit. AMPs have also been isolated from plants, for example, purothionin, obtained from *Triticum aestivum*, is effective against fungi and some phytopathogenic bacteria (Balls et al. 1942; De Caleya et al. 1972). The field of AMP research expanded further with the contribution of Hans Boman, Michael Zasloff, and Robert Lehrer, who autonomously identified and purified insect cecropins, amphibian magainins, and mammalian defensins, respectively (Steiner et al. 1981; Zasloff 1987; Ganz and Lehrer 1994). Over the last few years, a number of unconventional AMPs have also been discovered that are proteolytically processed from larger and functionally different proteins usually following a microbial infection (Bulet et al. 2004; Reverter et al. 2018). Although a lot of vertebrate antimicrobial peptides were discovered by the mid-1980s, it took yet another decade to discover the antimicrobial activity of fish peptides. In 1980, a

toxic peptide named pardaxin from Moses sole flatfish was characterized, but its antimicrobial activity was not observed until 1996 (Primor and Tu 1980; Oren and Shai 1996). Since then, a number of AMPs have been identified in fishes. AMP family characterized in fish includes piscidins which are homologous to cecropins, while other AMPs such as defensin, hepcidin, and cathelicidin have equivalent counterpart in vertebrates (Masso-Silva and Diamond 2014).

17.3 Fish Antimicrobial Peptide

As aquatic animals are rich source of AMPs, a myriad of them have been identified from these organisms. Many of these peptide families express in more than single species and cell type with different gene copy number in different fish species. AMPs have been isolated from mucus, skin surfaces, and mast cells of different aquatic organisms. The AMPs identified from fishes are reported to possess antibacterial, antiviral, antifungal, antiparasitic, and in some cases antitumor properties as well (Campagna et al. 2007; Falco et al. 2008; Chang et al. 2011). Most of the AMPs in fish are detected in early developmental stages with the highest expression level at post-fertilization for some and post-hatching for others, indicating the critical role of AMPs in protection against infections during embryonic development. Majority of the AMP mRNA transcripts expression are upregulated after bacterial infection, confirming their role in fish defense (Magnadóttir 2006; Milne et al. 2019). There are five major families of AMPs in fish and some peptides with no clear homology with any known bioactive peptides (Su 2011).

17.3.1 Piscidins

They are linear, amphipathic, α-helical antimicrobial peptides, widely distributed in various species of teleost fishes (Milne et al. 2019). Piscidin was first purified from the mast cells of hybrid striped bass (*Morone chrysops* (white bass) × *Morone saxatilis* (striped bass)) (Silphaduang et al. 2006). Their amino acid sequence length ranges from 18 to 46 residues, comprising a high proportion of basic amino acids (mainly histidine), phenylalanine, and isoleucine (Qiao et al. 2021). A broad-spectrum antibiotic activity of piscidins includes antibacterial, antifungal, antiviral, and antiparasitic properties (Colorni et al. 2008; Zahran and Noga 2010; Hu et al. 2019; Zheng et al. 2021). Members/paralogues, comprising piscidin family, are enlisted in Table 17.1.

In addition, there are also multiple structurally similar but functionally different piscidin isoforms identified in different fish species as well as within the same species. Among other piscidin isomers (i.e., Piscidin 1–7), Piscidin-1 shows the highest antibacterial activity, even against MRSA and has potential to permeabilize cancer cell membranes as well (Noga and Silphaduang 2003; Lin et al. 2012; Raju et al. 2020). LcP5L4, a piscidin-5 isoform isolated from *Larimichthys crocea*,

Table 17.1 Antimicrobial peptides in the piscidin family

AMPs	Length (amino acids)	Source	Expression site	Antimicrobial activity	References
Pleurocidin	25	*Pleuronectes americanus* (Winter flounder)	Skin, mucus cell, intestines	Gram positive (MRSA) Gram negative Anti-biofilm Yeast and Molds	Cole et al. (1997), Villalobos-Delgado et al. (2019) and Ko et al. (2019)
Misgurin	21	*Misgurnus anguillicaudatus* (Mudfish)	Whole body	Gram positive Gram negative Fungus	Park et al. (1997)
Chrysophsin-1 Chrysophsin-2 Chrysophsin-3	25 25 20	*Chrysophrys major* (Red sea bream)	Gills	Gram positive Gram negative	Mason et al. (2007) and Saitoh et al. (2019)
Moronecidin	22	*Morone chrysops* (White Bass)	Gill, skin, intestine, spleen, anterior kidney, and blood cells	Fungi, yeast Gram positive Gram negative Antibiotic-resistant bacteria like MRSA, *P. aeruginosa*, and vancomycin-resistant *E. faecalis*	Lauth et al. (2002)
Dicentracin	22	*Dicentrarchus labrax* (European sea bass)	Macrophages, granulocytes, and monocytes from head, kidney, peripheral blood, and peritoneal cavity	Antibacterial, antiviral, and antiparasitic activities	Salerno et al. (2007)
Epinecidin-1	25	*Epinephelus coioides* (Orange spotted grouper)	Head, kidneys, gills, liver, intestines, and skin	Antibacterial (both Gram positive and Gram negative), antifungal, antiviral, antiprotozoal,	Yin et al. (2006)

(continued)

Table 17.1 (continued)

AMPs	Length (amino acids)	Source	Expression site	Antimicrobial activity	References
				anticancer, immunomodulatory, and wound healing properties	
Myxinidin	12	*Myxine glutinosa* L. (Hagfish)	Epidermal mucus cavity	Gram positive and Gram negative with highest sensitivity shown by *A. salmonicida*, *L.* anguillarum, and *Y. ruckeri*	Subramanian et al. (2009)
Gaduscidins (GAD-1 and 2)	22 and 19	*Gadus morhua* (Atlantic cod)	Spleen, head kidney, peripheral blood, gill		Browne et al. (2011)
Chionodracine		*Chionodraco hamatus* (icefish)	Gills	Gram-positive and Gram-negative bacteria	Olivieri et al. (2015)
Trematocine		*Trematomus bernacchii* (red blooded Antarctic fish)	Head kidney, gills, lungs		Della Pelle et al. (2020)

showed antiparasitic activity against the parasite *Cryptocaryon irritans* (Zheng et al. 2021). Five piscidin isoforms have been identified from Nile tilapia *Oreochromis niloticus* (named TP1–5) (Peng et al. 2012) and six different piscidins from *Dicentrarchus labrax* that possess broad-spectrum antimicrobial activity (Barroso et al. 2020). A lot of studies have reported that piscidins exhibit strong activity against both fish and human bacterial pathogens, including some MDR-bacteria such as MRSA, vancomycin resistant *Enterococci* (VRE), etc. It interacts and disrupts the target cell membrane using toroidal pore mechanism (Falco et al. 2008).

17.3.2 Hepcidins

Hepcidin was first isolated from bacterially challenged hybrid striped bass, and since then, it has been screened in more than 40 teleost fish species (Shike et al. 2004). Mammalian, fish, and other predicted hepcidins share four to eight cysteine residues at conserved positions in N-terminal, which is crucial for both its optimal conformation and its antimicrobial activity (Hocquellet et al. 2012). The peptide has been detected in several tissues, and a high amount of hepcidin transcripts was rather found in acidophilic granulocytes of the spleen, heart, and stomach instead of the liver (Cuesta et al. 2008; Wang et al. 2009). Accumulating evidences have indicated that all fish hepcidin antimicrobial peptide (HAMP) isoforms can be categorized into two classes: HAMP1 and HAMP2. HAMP1 is more involved in iron regulation while HAMP2 in antimicrobial activity (Hilton and Lambert 2008; Mu et al. 2018; Neves et al. 2017). Hepcidin isolated from *Salmo caspius* (Caspian trout), having antimicrobial activity against *Streptococcus iniae* and *Aeromonas hydrophila*, belongs to HAMP2 class (Shirdel et al. 2019). Hepcidin from *Epinephelus coioides* shows rapid and potent inhibitory activity against *S. aureus* and *P. stutzeri* (Mohapatra et al. 2019). The teleost hepcidin is also found to be effective against protozoan parasitic infections caused by *Trypanosoma carassii* (Xie et al. 2019). Some hepcidins like PsHepcidin, from starry flounder *Platichthys stellatus*, show synergistic interaction with antibiotics and hence are used in combination with antibiotic therapy for the treatment of bacterial infections (Liu et al. 2018).

17.3.3 Defensins

Defensins are another cysteine-rich CAMP that are extensively distributed in nearly all life forms. Based upon the bonding pattern of conserved 6 cysteine residues to form intramolecular disulfide bonds, defensin family can be sub-categorized into α, β, and θ. Whilst α- and θ-defensins have only been found in mammals to date, the β-subfamily has a widespread distribution in all major vertebrate lineages from fish, amphibians, birds, reptiles, to mammals (Zou et al. 2007). In contrary to mammals, fish β-defensins are found to be encoded by three exons, producing a pro-peptide and a mature peptide with a signature motif of 6 conserved cysteine residues (Casadei et al. 2009). The peptide acts through interaction with bacterial membrane followed

by insertion and permeabilization by generating multiple pores in the membrane (Chaturvedi et al. 2015). The peptide is reported to be an effective antimicrobial with activity against G+ and G- bacteria, fungi, protozoa, and enveloped viruses (Chang et al. 2011; Contreras et al. 2020; Cuesta et al. 2011). ScBD, a beta-defensin type 2, isolated from mandarin fish *Siniperca chuatsi*, effectively inhibited *E. coli*, *S. aureus*, and *A. hydrophila* (Wang et al. 2012).

17.3.4 Cathelicidins

Cathelicidins are another class of antimicrobials, produced as pre-propeptides, which include a signal sequence and a highly conserved cathelin-like domain and a variable C-terminus antimicrobial domain (Tomasinsig and Zanetti 2005). Cathelicidin was first isolated from Atlantic hagfish *Myxine glutinosa*, and first jawed-fish cathelicidin, rtCATH_1, was identified from rainbow trout (Sun et al. 2007; Chang et al. 2005). It has been reported that CATH possess antibacterial activity against fish pathogen *V. anguillarum* with no hemolytic activity and also has a role in immunomodulation of fishes (Bridle et al. 2011). Cathelicidin also acts against a number of Gram-positive and Gram-negative bacteria by permeabilizing their lipid membranes (Uzzell et al. 2003). Fish cathelicidins are enriched with Arg, Gly, and Ser, forming β-sheet and/or random coil and exhibit no cytotoxic activities (Jiang et al. 2018; Maier et al. 2008). Based on the secondary structure, fish cathelicidins are roughly divided into two groups: peptides forming a disulfide bond and peptides forming an extended structure (Chen et al. 2019). Like other AMPs, gene copy number of cathelicidins also varies species to species (Maier et al. 2008; Zhang et al. 2015a, b).

17.4 Mechanism of Action

The mode of action of AMPs is one of the reasons for being popularly considered as a replacement over antibiotics. The secondary structure of AMPs, which is influenced by their microenvironment, is crucial for their antimicrobial activity. It has been reported that the α-helical structure is responsible for the salt-insensitivity of the peptide, as in the case of piscidins and some α-helical cathelicidins (Broekman et al. 2011). AMPs typically target the microbial cell membrane rather than a specific receptor. The cytoplasmic membrane of both Gram-positive and Gram-negative bacteria is rich in phospholipids with negatively charged head groups like phosphatidylglycerol, cardiolipin and phosphatidylserine. An additional electronegative charge is conferred on the outer leaflet due to the presence of teichoic acid in Gram-positive and LPS in Gram-negative bacterium (Gong et al. 2020). In contrast to bacteria, the outer leaflet phospholipid composition in higher vertebrates is predominantly of zwitterion molecules like phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, while the negatively charged head groups, if present, are found mostly in the inner leaflet of the membrane facing the cytoplasm

(Mahlapuu et al. 2016; Kumar et al. 2018). Thus, the positively charged AMP interacts with bacterial membrane selectively. After the initial interactions, the AMPs accumulate and then self-assemble on the bacterial membrane after reaching a certain concentration (Epand et al. 2016). AMPs thus embed themselves into the hydrophobic regions of the lipid membrane, thereby causing disintegration and permeabilization of the bacterial membrane, leading to leakage of cell contents and dissipation of transmembrane potential and/or pose multiple stress on the membrane proteins, ultimately leading to cell death (Bessin et al. 2004). There are three models explaining the action of AMPs on the target membranes: (1) carpet model, (2) barrel-stave model, and (3) toroidal-pore model.

The carpet model is a non-pore-forming type also known as the self-promoted uptake model. According to this technique, AMPs act by adsorbing parallel to the lipid bilayer of the target's membrane till it reaches a threshold concentration to cover the entire membrane surface, thus, forming a "carpet." Consequently, this unfavorable interaction leads to loss of membrane integrity eventually resulting in thinning of membrane bilayer, followed by disintegration of the membrane in the form of micelles, producing a detergent-like effect. The barrel-stave model is a trans-membrane pore type where the antimicrobials initially align parallel to the membrane and then insert in the bilayer in perpendicular manner, which facilitates lateral peptide-peptide interaction. The amphipathic structure of peptide is crucial for pore formation, where the hydrophobic region interacts with the membrane lipids and hydrophilic residues form the channel lumen. The toroidal-pore model is another trans-membrane pore formation type. In this, AMP insertion is similar to barrel-stave model; however, the specific peptide-bilayer interaction is absent. The aggregation of AMPs bends the membrane bilayer, and a pore is formed partly by the peptide and partly by phospholipid head groups. Some peptides are able to translocate to the cytoplasmic leaflet of the membrane and enter cytoplasm, thereby, targeting intracellular components (Epand et al. 2016; Dawood and Koshio 2016; Kumar et al. 2018).

17.5 Artificial Designing of AMPs

Many studies have reported the antimicrobial activity of natural AMPs. However, they are associated with several disadvantages such as cytotoxicity, low stability, and high cost of production (Hancock and Scott 2000). It is also reported that antimicrobial activity decreases with chain length and longer peptides are more cytotoxic (Dong et al. 2018). To overcome the problems associated with natural AMPs, many studies have been carried out to develop compositionally simple and short peptides through artificial designing (Hu et al. 2011; Kim et al. 2014; Qi et al. 2010). We have designed a short peptide of 12 residues using only 3 types of amino acids (RRWYRRWYRRWY). It was synthesized by solid-phase peptide synthesis using Fmoc chemistry. The peptide showed antimicrobial activity different fish bacteria such as *Edwardsiella tarda*, *Aeromonas sobria*, and *Vibrio parahaemolyticus* and is also potent against important oomycete, *Saprolegnia*

parasitica. The peptide was effective even against gentamicin and methicillin-resistant *Staphylococcus aureus*. The peptide showed stability at higher temperature and even in the presence of serum (Hussain Bhat et al. 2020). The main advantage of artificial designing and chemical synthesis of AMPs is the possibility to modify to make the peptide more stable, potent, less toxic to host cells, and less costly in terms of production due to shorter length (Bagheri et al. 2016; Carotenuto et al. 2008; Grieco et al. 2013). In the similar line, another peptide of 16 residues was designed and synthesized (Bhat et al. 2022). The peptide also produced antimicrobial effect against various pathogens and could inhibit the growth of *S. parasitica* in embryonated fish eggs. The peptide has less cytotoxic and hemolytic activity and retained its activity even in the presence of serum and salt. Findings in our studies indicate that artificially designed and chemically synthesized AMPs may serve as an alternative to antibiotic for combating bacterial and fungal pathogen encountered in aquaculture.

17.6 Production of AMPs

Isolation of AMPs from natural sources is not feasible enough to meet the rising demands of basic research and clinical trials. Therefore, exploring alternative strategies for large-scale production of AMPs is important for downstream application. For production of AMPs, two approaches, viz., chemical synthesis and recombinant technology, have been reported. Though chemical synthesis might prove to be economical, it can be done for peptide having a length up to 40 residues. Hence, heterologous expression is preferred for synthesis of longer peptides (Meng et al. 2021). The recombinant technology for AMP production offers less complicated, environment-friendly, and cost-effective method. Among various reported microbial systems, *E. coli* is one of the most widely used host systems. However, it poses some limitations such as contamination of final product with bacterial LPS. Other popular expression hosts are yeast *Pichia pastoris*, and now insect cells have also come up as an attractive option for AMP expression (Karbalaei et al. 2020; Käßer et al. 2022). Insect cells are used for insect AMP expression since they might pose toxicity to bacterial or fungal host. *P. pastoris* has been used for the low-cost production of Tilapia piscidin-4, Ch-penaeidins, and Mytichitin-CB (Meng et al. 2021; Li et al. 2005; Neshani and Eidgahi 2018). *E. coli* was used for large-scale production of myticusin-beta, Vpdef, and was studied for their potential as antibiotics and immunomodulatory molecule (Oh et al. 2020; Zhang et al. 2015a, b). Sathyan et al. (2012) reported that some of the peptides, obtained through recombinant approach, showed reduced potency compared to their chemically synthesized counterparts.

Many of the naturally occurring AMPs have been chemically synthesized for use in research to elucidate their biological activity. These synthetic AMPs were found to be as effective as naturally occurring ones, and in some cases, they gave better performance. Synthetic Fi-His1–21 showed significant inhibition of *V. vulnificus*, *P. aeruginosa*, *V. parahaemolyticus*, *V. cholerae*, and *S. aureus*. It also showed DNA-binding activity and anti-cancerous activity (Sruthy et al. 2019). Synthetic BsHep elevated the expressions of immune-relevant genes in liver of *Bostrychus*

sinensis and also improved its survival against *V. parahaemolyticus* infection (Shen et al. 2021).

17.7 Conclusion

A number of researches on AMPs are going on, leading to discovery of different peptides with antimicrobial property. With increase in comprehensive studies of AMPs at structural, functional, and genetic level, it is expected that soon AMPs will be commercialized as therapeutic replacement of antibiotics. In order to facilitate their commercial development, more attempts should be made on developing strategies to reduce the cost of production, to enhance stability, and to reduce toxicity to host cells. As isolation of AMPs from the natural source for application will be an expensive venture, heterologous expression may be considered. For development of short and effective AMPs, artificial designing and chemical synthesis may be advantageous.

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