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FISH EPIDERMAL MUCUS-DERIVED ANTIMICROBIAL PEPTIDES: CLASSIFICATION, STRUCTURE, BIOLOGICAL ACTIVITIES, AND POTENTIAL BIOTECHNOLOGICAL APPLICATIONS – A REVIEW

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Abstract

In recent years the advent and spread of diverse multidrug-resistant bacteria has become a serious concern not only in the medical field but also in public health. The dissemination of drug-resistant pathogens has been considerably hastened by human activities in the clinical practice, livestock, and aquaculture sectors. Therefore, there is an urgent need to explore novel classes of antimicrobial agents against resistant bacterial pathogens. Antimicrobial peptides (AMPs), or host defense peptides, are synthesized by ribosomal or non-ribosomal mechanisms, which are vital components of the innate immune system in fish. Fish epidermal mucus can serve as a source of unique peptides with a broad range of biological activities including antimicrobial activity towards multidrug-resistant pathogens, anticancer, wound healing, etc. AMPs derived from epidermal mucus are recognized as a novel class of antibiotics owing to their inimitable and multidimensional effects, including the low possibility for developing antimicrobial resistance, minimal cytotoxicity towards mammalian cells, high selective cytotoxicity against pathogens, low residual flesh, and the capacity to modulate the host immune responses. Additionally, the mucosal composition varies among fish species and is influenced by both endogenous and exogenous factors, which could be leveraged to identify new drugs in the future. This review investigates the AMPs produced by aquatic fish, their unique properties as potent therapeutic agents as well as classifications and biological activities.

Key words: antibiotic resistance, AMPs, bioactive compounds, fish skin mucus, mucus immunity

Although aquaculture is the fastest-growing agri-food sector, economic losses from infectious diseases, environmental pollution, and problems regarding antibiotic resistance limit its progress (Naiel et al., 2023; Wang et al., 2024). In most parts of the world, antibiotic application in the livestock industry outpaces its use in human medicine (Van Boeckel et al., 2015; Robinson et al., 2016) and this might occur similarly in aquaculture. In Chile, for instance, the use of fluoroquinolones in aquaculture has been more than 10 times their application as human medications in recent years (Rico et al., 2013; Cabello et al., 2016). In aquaculture, antibiotics are administered to fish principally in their diets and seldom as baths and injections for various purposes including treating, preventing, and/or controlling fish diseases (Markestad and Grave, 1997; Sørum, 2019). However, these chemical agents can influence both unhealthy and healthy fish (metaphylaxis) (Sørum, 2019). Besides, antibiotics from the uneaten feed (up to 30% in infected fish) remain in the sediment and/or dissolve in the water and eventually disperse in the environment (Björklund et al., 1990; Capone et al., 1996; Sapkota et al., 2008; Rodgers and Furones, 2009; Pelletier et al., 2009; Sørum, 2019). Furthermore, roughly 80% of the consumed antibiotics in fish can leak into the aquatic environments by the feces, urine, and other exudates (Björklund et al., 1990; Hektoen et al., 1995; Capone et al., 1996; Burka et al., 1997; Le and Munekage, 2004; Armstrong et al., 2005; Sørum, 2019). In this context, several studies have shown that remarkable concentrations of antibiotics remain biologically active in sediments for months to more than a year (Björklund et al., 1990; Capone et al., 1996; Coyne et al., 2001; Hektoen et al., 1995; Koeypudsa et al., 2005; Lai and Lin, 2009; Thi et al., 2008). Residual antibiotics have led to the emergence of antibiotic-resistant microbes among fish pathogens in the aquaculture industry (Cabello et al., 2013). Additionally, it influences a broad scope of wild fish and different aquatic animals, thereby reconstructing the microbiome of sediments and water by displacing susceptible microbial populations and different aquatic microorganisms with resistant ones (Cabello, 2006). It is also possible for antimicrobial-re-

sistance genes to spread to terrestrial species and human communities (Cabello et al., 2016). Some studies have shown that aquatic resources and fish microbiota are the origins of some antibiotic-resistant genes; for example, *tetC* gene in the *Chlamydia suis* genome, many plasmid-mediated quinolone resistance genes, and macrolide-resistance genes appear to have arisen from the genome of the salmon pathogen *Aeromonas salmonicida* (Roberts, 2009), *Klebsiella* and *Escherichia coli*, and *Photobacterium* and *Vibrio* species, respectively (Poirel et al., 2012; Aedo et al., 2014; Nonaka et al., 2015; Tomova et al., 2015).

In 2017, the World Health Organization (WHO) declared an urgent global call for a new kind of antibiotics (Willyard, 2017). Back in the pre-antibiotic era, WHO announced antimicrobial resistance as a global health emergency that would require more countries to invest in research topics related to antibiotic-resistant bacteria, especially for the priority pathogens not seen in the last century (Willyard, 2017). Worldwide deaths caused by antibiotic resistance are predicted to reach 10 million by 2050 (O' Neil, 2014; Hedmon et al., 2018). Therefore, it is crucial to explore and develop alternative antimicrobial agents on an urgent foundation. Antimicrobial peptides (AMPs) are a novel group of natural biomolecules that have been shown to exert innumerable benefits for significant global well-being (Naiel et al., 2023). They have become excellent candidates and have earned distinguished attention as novel antibiotics because of their high selective cytotoxicity against bacteria, low capacity to develop antimicrobial resistance, minimal cytotoxicity to mammalian cells, and modulation of host immune responses (Ebenhan et al., 2014; Mahmoodi-Khaledi et al., 2015). Additionally, AMPs exhibit antiviral and antifungal lytic activities, thereby expanding their antimicrobial potential and reducing the use of other chemotherapeutics. However, there are limited commercial AMP-based drugs available on the market.

Because many valuable reviews exist related to fish AMPs and their immunomodulatory activities (Valero et al., 2013; Masso-Silva and Diamond, 2014; Katzenback, 2015), in this study, we will not consider those subjects deeply. Although the general properties of AMPs are described in the first part of this review, the purpose of this review is to focus on epidermal mucus-derived antimicrobial peptides produced by aquatic fish, their classification, and biological activities.

History of AMPs

AMPs represent an immense class of extremely evolutionarily conserved peptides, which are discovered throughout nature, and exhibit a wide range of antimicrobial activities *in vitro* and *in vivo*. The discovery of AMPs dates back to the late 1920s and early 1930s. The most ancient and well-known AMP, nisin, is a cationic peptide with 34 amino acid residues, which was found in *Lactococcus lactis* in 1928 (Rogers, 1928). The second natural AMP exploration dates back to 1939, when Dubos discovered gramicidin, an antimicrobial component generated by *Bacillus brevis* from soil (Dubos, 1939). The first discovered vertebrate-derived AMP, defensin, was identified in leukocytes of rabbits in 1956 (Hirsch, 1956). In 1961, a 26-mer peptide originated from the venom of European honey bee (*Apis mellifera meda*) was melittin (Fischer and Neumann, 1961). Since 1957, extracellular fluids like blood, plasma, mucus, sweat, saliva, white blood cell secretions, and granule secretory vesicle extracts have been acclaimed for their antimicrobial characteristics (Skarnes and Watson, 1957). Nevertheless, it was not discovered until 1981 that the hemolymph (blood and plasma) of cecropia moth (*Hyalophora cecropia*) included AMPs known as cecropins (Steiner et al., 1981). Later, magainin was isolated and characterized by an amphibian (Zasloff, 1987). The discovery of AMPs in aquatic animals is relatively recent, with the first identification in the cutaneous mucus layer of the finless sole (*Pardachirus marmoratus*) in 1980 as a toxic peptide called pardaxin (Primor and Tu, 1980). However, its antimicrobial activity was not recognized until 1996 (Oren and Shai, 1996). Later, Cole et al. (1997) reported pleurocidin (Ple), originated from the winter flounder (*Pleuronectes americanus*) epidermal mucus. Since then, AMPs have been identified in all multicellular organisms, including mammals, plants, amphibians, reptiles, birds, fish, insects, protozoans, fungi, and prokaryotic organisms, facilitated by advancements in molecular biology and bioinformatics (Okella et al., 2020; Toldrá and Mora, 2022). To date, over 8,000 AMPs have been reported (http://www.bicnirrh.res.in/antimicrobial/). For more information on fish AMPs, refer to a recently published manuscript (Akhavan-Bahabadi et al., 2024 b).

Common characteristics of AMPs

In various examples, AMPs are encoded in the gene clusters and co-expressed, thereby many AMPs are accumulated in particular places (Lai and Gallo, 2009). AMPs are chiefly synthesized as inactive precursors, also called prepropeptides which their antimicrobial-active parts require a proteolytic process to discharge the bioactive molecules (Bals, 2000). Therefore, AMPs biological regulation does not only rely upon the expression level in the cells but also on access to proper proteases (Lai and Gallo, 2009). The proteolytic cleavage is dependent upon the tissue or cell character in which the AMPs are produced (Krause et al., 2000; Mookherjee and Hancock, 2007). In multicellular species, few AMPs are expressed constitutively, deposited in large amounts as inactive precursors in granules and discharged locally upon inflammation and infection circumstances, whereas the expression of others is begun in response to cytokines or pathogen-associated molecular patterns (PAMPs), and damage-associated molecular pattern (DAMP) (Chaturvedi et al., 2020; Hancock and Diamond, 2000; Lai and Gallo, 2009). Besides, post-translational changes like the C-terminal amidation, formation of disulfide bridges, and amino-terminus pyroglutamic acid formation can

provide the stability and action of a few AMPs (Krause et al., 2000). Nevertheless, it is reported to identify some unusual AMPs that are parts of proteins that display other known functions (Boman, 1995).

Albeit in respect of sequences, structures, and origins, AMPs from various origins have multiple and unique characteristics, AMPs are generally positively charged changing from +2 to +11 and may consist of a particular cationic part, but a small group of anionic peptides has been also identified and exhibited a particular action (Yeaman and Yount, 2003; Mahmoodi-Khaledi et al., 2015). This property is ascribed to the existence of specific amino acids (Arg and Lys, and sometimes His) (Kumar et al., 2018). Numerous reports have proven the relationship between the antimicrobial properties and the positive charge of AMPs (Dathe et al., 2001; Hong et al., 2001; Jiang et al., 2008; Lyu et al., 2016; Gagnon et al., 2017). For instance, if the charge distribution of magainin 2 increases from +3 to +5, the antibacterial effects on both gram-positive and -negative bacteria will be enhanced, but if this increase reaches up to +6 or +7, it will lead to the expansion of the hemolysis process and consequently the antimicrobial effects will be reduced or eliminated (Dathe et al., 2001). The omission of antimicrobial properties can be a result of an extraordinary interplay between the peptides and the phospholipid head groups that prevents the peptides from moving into the inner layer of the membrane (Yeaman and Yount, 2003).

Hydrophobicity is another staple feature for most of the AMPs and refers to the presence of the hydrophobic amino acid residues such as Met, Val, Leu, Ile, Trp, Phe, Ala, and Tyr in the peptide composition (typically 50%). Hydrophobicity indicates the ability of water-soluble peptides to dispense into the cytoplasmic membrane. It is requisite for lipid bilayer membrane permeabilization, while a high peptide hydrophobicity can be cytotoxic towards mammalian cells due to a weak antimicrobial selectivity (Chen et al., 2007; Yeaman and Yount, 2003; Yin et al., 2012). Similarly, Chen et al. (2007) found that a threshold hydrophobicity is necessary for an appropriate antimicrobial effect. Therefore, AMPs with less or more hydrophobicities than the reported optimal, made the peptides ineffective (Chen et al., 2007). Whenever the hydrophobicity is high, the access of peptides to the microbial membrane may be inhibited due to an increase in the possibility of dimerization (Kumar et al., 2018). Furthermore, the increased hydrophobicity of the nonpolar side of the amphipathic α-helical peptides leads to an increase in the lysis of red blood cells (RBC). This can be explained by the penetration of the hydrophobic core of the RBC membrane, which occurs due to the membrane affinity of AMPs with high hydrophobicity (Chen et al., 2007).

The third common property of AMPs is amphipathicity. It is defined as the relative abundance of hydrophobic and hydrophilic amino acid residues or domains inside a peptide. It can be also expressed as the hydrophobic and cationic amino acids equilibrium, not only at the primary structure but also concerning the two- or three-dimensional structure of the peptides. In regard to α-helical AMPs, interestingly, some studies demonstrated that interruption in the amphipathicity can result in an enhancement in the antimicrobial action and a reduction in RBC lysis (Chen et al., 2005; Hawrani et al., 2008; Jiang et al., 2011; Mihajlovic and Lazaridis, 2012; Takahashi et al., 2010). Additionally, both tachyplesin-1 and protegrin-1 possess beta-strand organization. In comparison to tachyplesin-1, protegrin-1 displays more amphipathic properties and the enhancement in amphipathicity can lead to a double increment in hemolytic effect, although it does not exert a significant effect on the antimicrobial action according to the interaction between amphipathicity and hemolytic activities (Edwards et al., 2016). Conversely, indolicidin analogs with high amphipathicity and charge exhibit weaker hemolysis, whereas they maintain antimicrobial effects (Falla and Hancock, 1997; Smirnova et al., 2004). These investigations recommend a complicated association between net charge, hydrophobicity, and amphipathicity in AMPs (Cashman-Kadri et al., 2022).

Mucus: the key component of the innate immune system

In teleosts, the thymus, head kidney, and pronephros constitute the primary lymphomyeloid organs, whereas secondary lymphomyeloid organs are the kidney, spleen, and mucosal-associated lymphatic tissue (MALT) (Beck and Peatman, 2015). Anatomically, MALT is designated as skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT), gut-associated lymphoid tissue (GALT), and nasal-associated lymphoid tissue (NALT) (Salinas et al., 2011; Das and Salinas, 2020; Yu et al., 2020). In contrast, the non-specific immune response comprises a set of defense pathways that protect a species from infection without any former exposure to infectious agents and is more important than the specific immune response for poikilothermic animals, including fish. It involves physical barriers as well as cellular and humoral responses (Valero et al., 2013). Non-specific responses in fish are considered indispensable elements in battling pathogenic microorganisms because of acquired immune system restrictions, their poikilothermic nature, their limited supply of immunoglobulins, and the slow proliferation, maturation and memory of B- and T-cells (Whyte, 2007). Hence, fishes rely heavily on innate immune components during ontogenesis. A fundamental non-specific immune component is the epidermal mucus layer on the teleost skin surface, which is secreted via mucous glands or goblet cells and serves as a natural, physical, biochemical, dynamic, and semi-permeable barrier between fish and its aquatic habitat (Shephard, 1993). It is significant as the first line of defense against the diverse range of invasive pathogens. Because the surrounding environment of fish is laden with opportunists or potential pathogens, many studies have shown that skin mucus is critical for the health of aquatic animals (Ellis, 2001; Rajanbabu and Chen, 2011). Moreover, the

viscous fluid on the surface of the fish body intervenes in numerous functions, including disease resistance, ionic and osmotic regulation, respiration, reproduction, locomotion, feeding, and communication (Negus, 1963; Ingram, 1980). The mucus layer is constantly produced and regularly sloughed off, which restrains the adherence of infectious agents and the long term settlement of potential pathogens and metazoan parasites (Nagashima et al., 2003). In addition, it is comprehended that fish skin mucus allocates several bioactive molecules that work as non-specific immune agents involving proteins and enzymes such as lysozyme, alkaline phosphatase (ALP), proteolytic enzymes, proteases, immunoglobulins, complements, lectins, C-reactive protein (CRP), transferrins and antimicrobial peptides and proteins (Subramanian et al., 2007, 2008 a; Palaksha et al., 2008; Paknejad et al., 2020; Bhatnagar and Rathi 2021; Hosseini Shekarabi et al., 2021; Dik et al., 2024).

Significance of fish epidermal mucus AMPs

Aquatic species comprise approximately half of the global biodiversity and fish as an extensive and heterogeneous collection of vertebrates with roughly 40,000 species that form a major part of the aquatic fauna, all of them secreting AMPs with great structural differences (Ángeles Esteban, 2012; Tiralongo et al., 2020). Fish epidermal mucus has numerous unique properties that convert it into a vital body's first line of defense against pathogens (Ángeles Esteban, 2012; Tiralongo et al., 2020). Therefore, they can be exploited to discover novel therapeutic agents in aquaculture and human diseases. The mucosal composition varies among fish species and is affected by endogenous (e.g., developmental stage, age, and sex) and exogenous elements (e.g., nutritional status, physicochemical factors, immunostimulants, infections, and stress) (Blackstock and Pickering, 1982; Zaccone et al., 1985; Tiralongo et al., 2020), which can be applied to identify novel drugs and tackle multidrug-resistant (MDR) pathogens.

The antimicrobial activity of fish epidermal mucus towards pathogens were proven over three decades ago (Austin and Mcintosh, 1988). This property has been observed in numerous fish species (Azadi et al., 2016; Ebran et al., 2000; Fouz et al., 1990; Hellio et al., 2002; Kanno et al., 1989; Lemaitre et al., 1996; Ramos et al., 2012; Subramanian et al., 2007, 2009; Tiralongo et al., 2020; Lirio et al., 2019). A recent study on the antimicrobial potential of skin mucus revealed that the acidic extract of skin mucus from each species exhibited effective antimicrobial activity against both gram-positive and gram-negative bacteria, comparable to the antibiotic fosfomycin (Ali et al., 2023 b). Additionally, peptide fractions with antimicrobial and antioxidant activities have been isolated from the epidermal exudates of Caspian sand gobies (*Neogobius fluviatilis pallasii*) (Akhavan-Bahabadi et al., 2020, 2021, 2024 a). Albeit, many amphibian and mammalian families have been studied to discover mucosal AMPs (Pukala et al., 2006; Dourado et al., 2007; Ming et al., 2007; McDonald et al., 2015), few studies have reported fish skin mucus-isolated AMPs (Table 1).

Fish epidermal mucus AMPs not only exhibit antibacterial activity but also demonstrate a wide array of additional beneficial properties. These include antiviral, antiparasitic, and antifungal activities, along with antitumor effects, antioxidant capabilities, and contributions to wound healing. Furthermore, they display anti-inflammatory and immunomodulatory properties, highlighting their diverse and valuable biological roles (Akhavan-Bahabadi et al., 2020, 2024 b; Fomina et al., 2020; Huang et al., 2015, 2014; Jung Kim et al., 2014).

Biofilm-related infections are resistant forms of pathogens and are considered significant medical issues, particularly due to the spread of multidrug resistance (MDR). According to the National Institute of Health (NIH), biofilm formation is associated with 65% of all microbial diseases and 80% of chronic illnesses (Zafer et al., 2024). It has been demonstrated that AMPs from fish epidermal mucus not only prevent biofilm formation but also disrupt existing biofilms. For instance, low concentrations of pleurocidin have been shown to both suppress biofilm formation and destroy preformed biofilms (Ko et al., 2019; Lin et al., 2022; Tao et al., 2011).

Available evidence also shows that fish phagocytic granulocytes can kill bacteria in phagosomes through the intracellular release of AMPs, such as piscidins, indicating that fish epidermal mucus-derived AMPs can exert their antibacterial role towards intracellular species (Mulero et al., 2008).

Fish epidermal mucus AMPs can maintain antimicrobial activity at high salt concentrations (salt-tolerant) (Cole et al., 1997; Lauth et al., 2002; Bergsson et al., 2005; Subramanian et al., 2009), high temperatures (heat-stable, heat-tolerant) (Cole et al., 1997; Fernandes et al., 2003; Liang et al., 2011; Sun et al., 2012), and exhibit relatively weak cytotoxicity and hemolytic activity in mammalian cells (Kim et al., 2010). For instance, pleurocidin exhibits the highest bactericidal activity (100%) at 300 mM NaCl and at salinity equal to that of marine conditions (625 mM), its activity is reduced to 40% (Cole et al., 1997). In addition, a histone H2B-derived peptide from Atlantic cod (*Gadus morhua*) skin exudates showed maximum antibacterial activity at 500 mM NaCl (Bergsson et al., 2005). Myxinidin maintains its activity as a bactericidal agent even at 300 mM NaCl concentration (Subramanian et al., 2009). In addition, the thermal stability of fish epidermal mucus-derived AMPs such as pleurocidin and histone H1 (boiling for only 5 min) (Cole et al., 1997; Richards et al., 2001), oncorhyncin III (after incubation at 80°C for 5 min) (Fernandes et al., 2003) and AJN-10 (after boiling for 10 min) (Liang et al., 2011) has been reported.

It has been proposed that bacterial death because of fish epidermal mucus-derived AMPs can be achieved through interactions with various targets or several peptides interacting with a target, which is defined as

a multi-hit mechanism, to enhance the function of AMPs and prevent resistance development (Nguyen et al., 2011; Peschel and Sahl, 2006; Wang et al., 2022; Yeaman and Yount, 2003; Zhang et al., 2000). For instance, pleurocidin penetrates *E. coli* without destroying its plasma membrane and hinders its intracellular function by binding to RNA and DNA (Patrzykat et al., 2002; Manzo et al., 2020). These observations suggest that the biological activity of fish epidermal mucus proteins is a suitable reservoir of unique AMPs for both fish and human health-related utilization.

Furthermore, fish skin offers great opportunities to consider the evolutionary roots of human innate antimicrobial defensive systems, as several of these protective molecules are similar between fish and human skin, and AMPs have been evolutionarily preserved (Rakers et al., 2010). In addition, fish mucus could serve as a great source of AMPs because it is discarded by fisheries or aquaculture industries. The isolation of AMPs from fish skin mucus or their synthetic/recombinant production could provide great opportunities for the biotechnology industry related to blue growth strategies, which could be applied to industry or biomedicine. Therefore, understanding the regulation, production, and function of fish skin mucus AMPs could result in a better understanding of AMP regulation in humans and their biotechnological applications.

Classification of fish AMPs

AMPs belong to five subgroups based on their secondary structure: (1) linear amphipathic alpha-helix peptides, (2) specific amino acid-laden peptides, (3) larger protein-derived peptides, (4) cyclic cysteine-rich AMPs, and (5) other peptides with distinct conformations.

Cationic AMPs

Linear, amphipathic α-helical AMPs

The first subgroup comprises cationic, α-helical, linear, non-cysteine and amphipathic AMPs which are derived from several sources, including fish, amphibians, insects, nematodes, and mammals except humans (Rana et al., 2006; Yount et al., 2006; Raju et al., 2021). They are the main components of the vertebrate skin.

Although nearly all these AMPs exist as unstructured or flexible extended structures in aqueous environments, they adopt a fixed amphipathic structure once they come into contact with biological membranes (Valero et al., 2013). This common structure allows AMPs to rapidly permeabilize the membranes of pathogenic microorganisms via toroidal pore formation (Oren and Shai, 1996; Campagna et al., 2007). In this model, AMPs insert vertically into the membrane, causing a limited curvature in the membrane region where pores are partially formed by the phospholipid head groups and, to a lesser extent, by the AMPs themselves. The mechanisms of action of fish AMPs have been discussed in detail by Akhavan-Bahabadi et al. (2024 b). This leads to membrane dispersion and the release of intracellular materials. However, studies using different membrane models have shown that the membrane composition is a significant factor in the lytic potential of piscidin action (Rahmanpour et al., 2013). Furthermore, it has been displayed that piscidin-1 and -3 cause AMP-membrane interactions via the vertical orientation of the α-helical in the lipid bilayer model surfaces (Chekmenev et al., 2006; Mason et al., 2007). However, piscidin does not cause severe rupture of the bacterial membrane at inhibitory concentrations and can inhibit macromolecular synthesis (Patrzykat et al., 2002).

Members of the piscidin family exhibit robust biological activity against a wide range of microorganisms. They are highly active against gram-positive and gram-negative bacteria (Bae et al., 2014; Chiou et al., 2006; Cole et al., 1997; Lauth et al., 2002; Mohammadi et al., 2021; Noga et al., 2009; Peng et al., 2012; Qiao et al., 2021; Ruangsri et al., 2012). Besides, piscidins have antiparasitic (Colorni et al., 2008; Dezfuli et al., 2008; Lin et al., 2009; Niu et al., 2013; Park et al., 2011; Ruangsri et al., 2012), antifungal (Cho and Lee, 2011; Jung et al., 2007; Niu et al., 2013; Sung et al., 2008; Sung and Lee, 2008), antiviral (Chinchar et al., 2004; Raju et al., 2021; Ali et al., 2022; Dik et al., 2024), and antitumor activities (Chen et al., 2009; Han et al., 2016 b; Hiemstra and Zaat, 2013; Hilchie et al., 2011; Lin et al., 2009, 2013; Uen et al., 2019). It has also been shown that piscidins can accompany other AMPs to increase their activity (Cole et al., 2000; Patrzykat et al., 2001; Wang et al., 2010).

Pardaxin

Pardaxin is a cationic, amphipathic, pore-forming polypeptide neurotoxin that originates from the skin secretions of the Red Sea flatfish (*Pardachirus marmoratus*). It was first identified in early 1980s (Primor and Tu, 1980), although its antimicrobial activity was not recognized until 1996 (Oren and Shai, 1996). Three analogous peptides were found in *P. pavoninus* and were sequenced (Thompson et al., 1986), named Pa1, Pa2, and Pa3. Another peptide was identified in *P. marmoratus*, named Pa5 (Adermann et al., 1998), whereas the first pardaxin found in *P. marmoratus* was named Pa4 (Primor and Tu, 1980). These peptides have a highly homologous sequence, differing only at positions 5, 14, and 31 (Thompson et al., 1986; Cipolari et al., 2020) (Table 1). All five peptides involve a single 33 amino acids-polypeptide chain (Thompson et al., 1986) that begins with glycine (G) and ends with glutamic acid (E); therefore, it is also referred to as GE33 (Oren and Shai, 1996). GE33 has strong antibacterial activity towards both gram-negative and gram-positive bacteria, employing disruption of the membrane, with great hemolytic activity against human red blood cells (Oren and Shai, 1996). In addition, pardaxin has been confirmed to have diverse neurotoxic potentials and robust antifungal and antiviral activities (Lelkes and Lazarovici, 1988; Pal et al., 1981 a, b; Thennarasu and Nagaraj, 1996). Synthetic pardaxin also presents biological activities similar to those of the natural form (Shai

et al., 1988), with the capability pore formation on lipid membranes (Lelkes and Lazarovici, 1988).

Numerous studies have investigated the mode of action of pardaxin (Shai et al., 1990; Oren and Shai, 1996; Shai, 2002) and the influence of factors such as lipid composition and pH (Rapaport and Shai, 1991; Epand et al., 2012). Pardaxin possesses a helix–hinge–helix structure, a conventional structural motif observed in both cytotoxic and antibacterial peptides. Pardaxin NMR spectroscopy revealed that the membrane construction affected the orientation of the peptide. It further disclosed that the lipid bilayers formed entirely of zwitterionic lipids have been disrupted considerably by the peptide (Hallock et al., 2002). Moreover, it has been shown that eliminating the pardaxin carboxyl-terminus domain significantly reduces the hemolysis, and the aminated pardaxin was dramatically more efficient than the whole form of pardaxin against most bacteria, submitting that the pardaxin's nonselective action versus bacteria and red blood cells is related to the carboxyl-terminus domain (Oren and Shai, 1996). In addition, pardaxin stimulates a dopamine-releasing factor and arachidonic acid cascade in the host via extracellular signal-regulated kinase (ERK) and other signaling pathways (Abu-Raya et al., 1998, 1999; Bloch-Shilderman et al., 2001).

Pardaxin has displayed potential to affect various cancer cells viability including HT-1080 (Huang et al., 2011), HeLa (Hsu et al., 2011), human fibrosarcoma (Huang and Chen, 2013), murine fibrosarcoma (Wu et al., 2012), and bladder-associated tumor (Huang et al., 2013), canine perianal gland adenoma (Pan et al., 2015), oral squamous cell carcinoma (Han et al., 2016 b), leukemia,THP-1, and U937 cells (Uen et al., 2019).

In fibrosarcoma HT1080 cells, GE33 activates caspase-3 and caspase-7, interrupts the mitochondrial membrane, and results in the accumulation of reactive oxygen species (ROS) (Huang et al., 2011). Moreover, the anticancer activity of pardaxin is mediated by programmed cell death (apoptosis) in a dose-dependent manner (Hsu et al., 2011). Increases in the subG1 phase and caspase-8 activity, as well as DNA fragmentation, have been proposed to cause HeLa cell death via apoptosis (Wu et al., 2012). *In vitro* and *in vivo* antitumor activities towards murine fibrosarcoma have been investigated (Wu et al., 2012). In tumor-bearing mice, GE33 activates caspase-7, hinders the propagation of the MN-11 cells, and initiates apoptosis by the death receptor/nuclear factor (NF)-κB signaling mechanism. It has been also reflected that the intratumoral injection of pardaxin into mice considerably repressed tumor growth (Wu et al., 2012). Proteomic studies have also shown that pardaxin acts by inducing the phosphorylation of c-Jun, the unfolded protein response (UPR), and that oxidative stress triggers both caspase- and AIF-dependent apoptotic mechanisms (Huang and Chen, 2013). In HeLa cells, the AP-1 (activator protein-1) subunit c-Jun induces pardaxin-induced cell death (Huang and Chen, 2013). Transcriptome investigation in pardaxin-exposed HT-1080 cells showed upregulation of additional genes encoding subunits in AP-1, including JUND, c-FOS, and JUNB (Ting et al., 2014). Pardaxin-exposed cells show an increase in cellular calcium levels, although the obstruction of cellular calcium signaling disturbs pardaxin-induced cell death (Ting et al., 2014). The potential of pardaxin as a cancer vaccine in mouse tumor specimens was examined, and it was confirmed that murine bladder carcinoma cell (MBT-2) proliferation *in vitro* was stopped in the presence of pardaxin (Ting et al., 2014). GE33, accompanied by inactivated MBT-2 lysate, improves nitric oxide (NO) excretion and alters MCP-1, IL-6, and IL-12 levels in a mouse macrophage cell line (Ting et al., 2014). In addition, immunization of mice arrested the progression of MBT-2 cell-mediated tumors and heightened the activation of natural killer (NK) cells, T-cell receptors and cytotoxic T cells. Immunization expands the recruitment of lymphocytes, monocytes, NK cells and T-helper cells while diminishing the expression of vascular endothelial growth factor (Huang et al., 2013). GE33 represses the extension of oral squamous cell carcinoma (SCC4) cells and induces apoptosis by upregulation of caspase-3 activity (Wang et al., 2010). Moreover, cell cycle analysis revealed that GE33 reduced the G2/M phase and inhibited the proliferation of OSCC (oral squamous cell carcinoma) cells, presumably by hindering the expression of cyclin B1 and increasing p53 expression (Han et al., 2016 b). Pardaxin considerably suppressed the viability of leukemic cells (THP-1 and U937 cells) and prevented the cell cycle G0/G1 phase in the cells. Pardaxin further results in the differentiation and maturation of leukemic cells into macrophage-like cells that possess phagocytic strength. Furthermore, pardaxin upregulates the expression of MyD88 in THP-1 and U937 cells (Uen et al., 2019).

The potential of pardaxin to support the healing of contaminated wounds in mice was investigated. Pardaxin-treated methicillin-resistant *Staphylococcus aureus* (MRSA) mice have significantly lower bacterial counts and improved wound closure (Huang et al., 2014). Compared with vancomycin-treated mice, pardaxin-exposed mice showed accelerated re-epithelialization and dermal maturation. Additionally, pardaxin treatment managed the excessive recruitment of macrophages and monocytes and elevated VEGF expression. The studies have shown that pardaxin is an AMP with noteworthy potential for becoming a promising therapeutic agent (Huang et al., 2014).

Recently, it has been suggested that pardaxin could be a potential candidate for osteoporosis treatment due to its regulation of the osteogenesis pathway (Lee et al., 2022).

Pleurocidin

Pleurocidin is a 25-mer cationic, linear, amphipathic, salt resistant and highly basic AMP (pI=10.02) that was initially isolated from the skin-secreted mucus of winter flounder (*Pseudopleuronectes americanus*) (Cole et al., 1997; McMillan and Coombs, 2021), but 20 Ple-family cationic AMPs (NRC-1 to -20) were found in numerous

Atlantic flounder species and studied for their biological activities in the early twenty-first century (Patrzykat et al., 2003). In addition, Douglas et al. (2001, 2003 a, b) discovered four genes and three pseudogenes of pleurocidin in the winter flounder. The Ple gene is composed of four exons and three introns and encodes a 68 residue amino acids-precursor (Cole et al., 2000). Ple is found in general epithelial goblet cells (Cole et al., 1997), mucus-producing cells of the intestine (Cole et al., 2000), and circulating cells resembling mastocytes in winter flounder (Murray et al., 2003), whereas GE33 is located only in specific mucous cells covering the anal and dorsal fins of the Red Sea Moses sole (*Pardachirus marmoratus*) (Clark, 1974).

Ple exhibits antimicrobial activity towards a wide variety of gram-positive and gram-negative species, particularly against drug-resistant *S. aureus*, cariogenic pathogens, and the human opportunistic oral pathogen *Candida albicans*, with no cytotoxicity and weak hemolytic effects on mammalian cells (Cole et al., 1997, 2000; Douglas et al., 2003 a, b; Patrzykat et al., 2003; Mason et al., 2006; Jung et al., 2007; Lee et al., 2009 b; Tao et al., 2011; Ko et al., 2019; McMillan and Coombs, 2021). Ple dissipates to the plasma membrane of bacterial cells and may target intracellular functions. Briefly, it communicates with the negatively charged parts of microorganisms through electrostatic interactions and provides an antibacterial effect by generating transitory pores in the bacterial membrane, enabling Ple to enter the bacteria and adhere to DNA, interfering with intracellular functions (Saint et al., 2002; Ko et al., 2019). For the first time, it was also revealed that AMPs can change their antimicrobial mechanism depending on their concentration. Lee and Lee (2016) suggested that a sub-cytotoxic dose of Ple caused intracellular apoptosis-like death (ALD) in *E. coli*, which was induced through caspase-like protein and RecA activation (in bacteria, the SOS response is a major stress response system to DNA damage, mediated by the activation of *RecA*, which acts similarly to caspases and leads to ALD). In addition, it has been reported that Ple plays a fungicidal role through accumulation on the membrane, rupture of the integrity of the fungal membrane, or induction of apoptosis by the accumulation of ROS in cells (Cho and Lee, 2011, 2013; Jung et al., 2007). Concerning antimicrobial activity, studies have shown that hydrophobicity and the amino-terminus part of Ple play more fundamental roles than other principal factors (Lee and Lee, 2010; Cho et al., 2012; Choi and Lee, 2013).

Because pathogenic biofilms develop resistance to antibiotics, the effect of Ple on biofilm-associated infections has been investigated (Tao et al., 2011; Ko et al., 2019). The results showed that low concentrations of Ple not only could prevent the formation of biofilms but also destroy the formed biofilms (Tao et al., 2011; Ko et al., 2019). In addition, the effects of Ple were preserved in the presence of physiological NaCl concentrations (Ko et al., 2019).

The immunomodulatory function of Ple has been also confirmed. Pundir et al. (2014) showed that Ple NRC-04 activates human mast cells by binding to and recruiting the N-formyl-peptide receptor 1 receptor (FPRL1) Ple-induced activation of mast cells initiates degranulation and liberation of preformed granule-containing mediators and stimulates the generation of chemokines (Lee and Lee, 2008). The discharge of mast cell mediators can recruit and activate inflammatory cells such as eosinophils and T cells, and probably participate in natural and acquired immune responses (Pundir et al., 2014).

In vitro and *in vivo* anticancer action of Ple has been confirmed by discriminately impairing or preventing the growth and lysis of a broad category of breast carcinoma-originated cell lines, including drug-resistant variants, such as MCF7-TX400 and SKBR3 cells (Hilchie et al., 2011), various mammalian myeloma cells (Hilchie et al., 2013), and leukemia cells (Morash et al., 2011). However, it displays weak or no lytic activity towards human cells such as umbilical vein endothelial cells, dermal fibroblasts, or red blood cells (Hilchie et al., 2011). For example, sub-cytotoxic doses of both NRC-03 and NRC-07 improve breast cancer cell damage by substantially decreasing the median effective concentration (EC50) of numerous cytotoxic compounds such as docetaxel and cisplatin (Hilchie et al., 2011). Hilchie et al. (2011) explained that NRC-03- and NRC-07-induced cell death was caused by the expression of negatively charged components on the surface of breast tumor cells, implying that the destruction of breast cancer cells begins via peptide binding to anionically charged cell-surface components of breast carcinoma cells leading to notable and irreversible loss of the cytoplasmic membrane (Hilchie et al., 2011). Moreover, intratumoral injection of NRC-03 and NRC-07 hindered the growth of breast carcinoma xenografts in immuno-deficient mice (Hilchie et al., 2011). It has also been demonstrated that NRC-03 exhibits stronger binding to various human and mouse myeloma cells and a greater cytotoxic effect than NRC-07 by inflicting widespread membrane damage and DNA cleavage (Hilchie et al., 2013). In addition, intratumoral treatment with NRC-03 interfered with the growth of multiple myeloma xenografts in non-obese diabetic/severe combined immunodeficiency (Hilchie et al., 2013), indicating its potential application as a chemosensitizing factor.

Nevertheless, the potential therapeutic advantages of AMPs may be associated with proteolytic degradation, and toxicity, and several efforts have been made to improve their bioactivity and biocompatibility, such as the substitution of D-amino acids and some amino acids for proteolytic resistance and the reduction of cytotoxic and/or hemolytic effects (Hong et al., 1999; Kumar et al., 2018). For example, low degradation of NRC-03 by trypsin and limited NRC-03-induced cytotoxicity in the presence of serum have been reported (Hilchie et al., 2011). However, concerning both stability and capability, synthetic [D]-NRC-03 enhanced the lysis of breast tumor

cell xenografts in immuno-deficient mice and was not degraded by trypsin or human serum, although it exhibited greater cytotoxicity than NRC-03 towards various varieties of normal cells (Hilchie et al., 2015).

In addition, D-Ple, in comparison with L-Ple, presented slightly enhanced antifungal activity and notably increased proteolytic cleavage towards various serum proteases (Jung et al., 2007). In contrast, Lee and Lee (2008) reported that the substitution of D-amino acid (D-Ple) led to diminished antibacterial potency and exceptionally decreased hemolytic activity towards human red blood cells. Also, it has been shown to limit the hydrophobicity, hemolytic activity, and α-helical structure of analog peptides designed through the substitution of Arg or Ser at the hydrophobic face of Ple, without any influence on the antifungal actions (Sung and Lee, 2008).

Myxinidin

Myxinidin is a 12-mer, cationic (with a net charge of +2), salt-resistant AMP that was originally derived from the cutaneous mucus layer of hagfish (Subramanian et al., 2009). It represents the robust antimicrobial effect towards numerous fish and human pathogenic species (Subramanian et al., 2008 b). Myxinidin has an approximate molecular weight of 1.3 kDa, is one of the shortest natural AMPs found until now that exhibited antibacterial activity to a wide variety of pathogenic gram-positive and gram-negative bacteria, including multidrug-resistant strains (Subramanian et al., 2009; Cantisani et al., 2013, 2014; Han et al., 2016 a, 2017; Lombardi et al., 2017), and the fungicidal action towards yeast pathogenic species with no lytic activity towards mammalian red blood cells (Subramanian et al., 2009). Additionally, it retains its antimicrobial functions as the NaCl concentration in the environment increases.

Myxinidin can incorporate into bacterial bilayer membranes due to the α-helical structure to create ion channels or pores that cause cell rupture and death (Subramanian et al., 2009; Han et al., 2016 a). However, the reports have shown that the secondary conformation of AMPs is dependent on the lipid membrane composition (Cantisani et al., 2013, 2014; Han et al., 2016 a; Lombardi et al., 2017). In complex with bacterial bilayers made up of phosphatidylglycerol (PG) or phosphatidylethanolamine (PE):PG, the structurally designed myxinidin analogs displayed α-helical and β-strand secondary organizations (Cantisani et al., 2013); nevertheless, they presented a random coil structure in the lipid bilayers models mimicking red blood cells [phosphatidylcholine (PC) : cholesterol (CH)] and mammalian cells [(PC, PC: sphingomyelin (SM), SM)] (Han et al., 2016 a). On the other hand, it has been revealed that the higher amount of anionic cardiolipin (CL)-comprising bacterial species such as *Pseudomonas aeruginosa* and *Salmonella typhimurium* displayed less susceptibility to myxinidin in comparison to WMR, one of the myxinidin analogs (Cantisani et al., 2013, 2014). Further investigation of antimicrobial action of these peptides by different biomembrane models confirmed the fundamental role of CL to bind and disintegrate the membrane (Lombardi et al., 2017), as WMR leads to large structural dissipation of the DOPE/DOPG/CL (*P. aeruginosa*) bilayer, whereas myxinidin communicates better with the DOPE/DOPG (*E. coli*) membrane (Lombardi et al., 2017).

Investigation of myxinidin structure-function associations demonstrated that although the capacity to adopt a helix organization in complex with cytoplasmic membranes is a key factor for inactivation of pathogenic species, various other agents like the number of positive charge amino acids, the helical-stabilizing (such as Lys, Leu, and Ala) or destabilizing (such as Pro) amino acid residues, the nonpolar side disruption, and the effects of replacements upon the helical dipole appear to serve a crucial function (Cantisani et al., 2013, 2014). In other words, a proper equilibrium between amphipathicity, hydrophobicity, and cationic charge in α-helical analogs of this peptide represented the principal role in the optimization of their antibacterial and hemolytic capabilities (Cantisani et al., 2014). However, some studies revealed that, in addition to net positive charge, the type and the position of cationic amino acids are essential determinants in therapeutic applications of designed myxinidin analogs (Cantisani et al., 2013). For example, Cantisani et al. (2014) designed the mutated peptides with a notable improvement in the antibacterial potency through inserting a Trp in the amino terminus and by the concurrent change in residues present in positions 3, 4, and 11 with Arg (Cantisani et al., 2014). Similar results have been reported regarding Trp amino acid. The antimicrobial capability of the myxinidin analogs enhanced by the presence of Trp amino acid residues at positions 1 and 4 from their amino terminus sequence, whereas the Trp amino acid residues at positions 1 and 2 from the carboxyl terminus sequence of the peptidic molecules caused their effect stop (Cantisani et al., 2013).

In another study antimicrobial potency of myxinidin mutants created by substituting principal amino acid residues with Trp in the hydrophobic side and Lys, Arg in the hydrophilic side was investigated (Han et al., 2016 a). Findings showed that myxinidin2 (Lys-comprising peptide) and myxinidin3 (Arg-comprising peptide) both displayed a robust antimicrobial action towards both gram-positive and gram-negative bacteria, including multidrug-resistant strains and also block bacterial biofilm formation even in the presence of NaCl and in presence of gelatin (Han et al., 2016 a, 2017) with little or no hemolytic or cytotoxic effects at active concentrations towards human red blood cells (hRBCs) and normal human keratinocytes (NHKs), respectively (Han et al., 2016 a).

Moreover, myxinidin2 and myxinidin3 exhibited strong wound-closure action both *in vitro* and *in vivo* (Han et al., 2017). These peptidic molecules accelerated infected wound healing through mitigating inflammatory responses by the reduction in infiltration of IL-6, IL-8, and tumor necrosis factor (TNF)-α as well as modulation of downstream mediators like signal transducer and acti-

vator of transcription (STAT3), p38, Jun-amino-terminal kinase (JNK) and NF-κB, and also increased the re-epithelialization of wounds contaminated with antibiotic-resistant species by stimulation of the strong migration and proliferation of primary keratinocytes (NHKs) *in vitro*, which includes transactivation of epidermal growth factor receptor (EGFR) (Han et al., 2017). In BALB/c mice model contaminated with antibiotic-resistant species, both myxinidin2 and myxinidin3 eliminated the infection and improved wound healing. Besides, histological investigations revealed that these peptides inhibited skin necrosis and decreased inflammatory cell secretion (Han et al., 2017). Furthermore, Mohanram and Bhattacharjya (2016) designed myxinidin analogues that exhibited strong antibacterial activity in moderately saline water (up to 0.3 M NaCl) and human serum conditions, but had a weak lytic effect on red blood cells (Mohanram and Bhattacharjya, 2016).

Grammistins

Grammistins are cationic, linear, highly amphiphilic alpha-helix toxic peptides with the membrane-lytic antibacterial potency that was originally founded in the epithelial mucosal layer of the spotted soapfish (*Pogonoperca punctata*) near a half-century ago (Hashimoto and Oshima, 1972; Shiomi et al., 2000, 2001; Yokota et al., 2001). However, to date, 13 grammistins have been derived from the epidermal exudates of the golden-striped soapfish (*Grammistes sexlineatus*) (Gs A, Gs B, Gs C, Gs D, Gs E, Gs 1, and Gs 2) and *P. punctate* (Pp 1 = Gs D, Pp 2a, Pp 2b = Gs E, Pp 3, Pp 4a = Gs 2, and Pp 4b) (Hashimoto and Oshima, 1972; Oshima et al., 1974; Oshima and Hashimoto, 1976; Shiomi et al., 2000, 2001; Sugiyama et al., 2005).

The grammistins are fallen into 3 classes according to the sequence and length: class I (Gs 1, Pp 4a = Gs 2, Pp 4b and Pp A) formed of 24 or 25 amino acid residues, class II (Gs B, Pp 1 = Gs D, Pp 2a, Pp 2b = Gs E and Pp B) of 12 or 13 amino acid residues, and class III (Gs A, Gs C, Pp 3 and Pp C) of 25–28 amino acid residues (Sugiyama et al., 2005; Cipolari et al., 2020).

Grammistin-like toxins have also been identified in the cutaneous mucus layer of coral-gobies of the genus *Gobiodon* (Oshima et al., 1974; Shiomi and Hashimoto, 1975) and the clingfish (*Diademichthys lineatus*) (Hori et al., 1979).

All the known grammistins are lethal towards a wide variety of bacterial groups including both gram-positive and gram-negative species, and they also possess hemolytic and ichthyotoxic capacities except for Gs A, Gs B, and Gs C (Shiomi et al., 2001; Yokota et al., 2001; Sugiyama et al., 2005, 2006; Kaji et al., 2006). Like the two groups of peptide toxins (melittin, and pardaxins), grammistins also play their functions through aggregating as multimeric molecules comprising 3–4 peptides, incorporating into the microbial membrane and creating pores (Shiomi et al., 2000). The investigations revealed that the amino-terminal Phe amino acid and the amphiphilic alpha-helical conformation are essential for the hemolytic activity of grammistins and being cationic is the main characteristic for the antibacterial actions (Sugiyama et al., 2005, 2006).

The hemolytic screening of a cDNA phage library from *P. punctata* skin revealed the presence of 6 grammistins, containing a highly conserved targeting signal that includes a pair of the basic amino acid (Lys-Arg) at multiple locations including the carboxyl-terminus (Kaji et al., 2006).

Pelteobagrin

A novel cationic, linear amphipathic alpha-helical 20-mer AMP, pelteobagrin, with the molecular mass and pI value approximately 2.2 kDa and 10, respectively, has been derived from the epidermal-secreted mucus of yellow catfish, *Pelteobagrus fulvidraco* that bears no resemblance to known AMPs (Su, 2011). It has been shown that pelteobagrin fulfills a wide range of antibacterial activity towards gram-positive (*Bacillus subtilis* and *S. aureus*) and gram-negative (*E. coli*) species as well as fungi (*C. albicans*) with no toxicity to rabbit red blood cells. Besides, its activity is moderately salt-resistant (up to 137 mM). Pelteobagrin removes bacterial pathogenic species through the effect on both the cell wall and the plasma membrane (Su, 2011). Recently, seven novel cationic, short antimicrobial peptides (11–16 mer) have been isolated from the skin mucus of African catfish, *Clarias gariepinus* (Okella et al., 2021). The peptides had a low molecular weight (1–1.6 KDa) and exerted more than 54% of hydrophobicity. The four of these AMPs represent α-helix structuring and render them amphipathic. These peptides serve antibacterial activities toward gram-positive (*S. aureus*) and gram-negative (*E. coli*) species (Okella et al., 2021).

Specific amino acids-laden AMPs

The AMPs in this group are linear and loaded with distinct amino acids without cysteine residues. Members belong to a group comprising (1) proline-loaded AMPs, (2) tryptophan-loaded AMPs, and (3) arginine–proline-loaded AMPs (Brogden, 2005). Focusing on aquatic organisms, a proline-laden 6.5 kD AMP isolated from the hemocytes of the shore crab, *Carcinus maenas* (Schnapp et al., 1996) and astacidin-2, a 14-residue, proline, and arginine-laden AMP, with an approximate molecular mass of 1.8 kDa derived from the hemocytes of the signal crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2007), are the only identified representatives of these categories.

Larger proteins-originated AMPs

The group is composed of AMPs that are parts of or originate from larger proteins (Table 1), which are similar in length and structure to other AMPs. In relation to AMPs from fish and shellfish, this group is divided into five subgroups: (1) AMPs isolated from various histone proteins (H), (2) AMPs originated from intracellular pro-

teins, (3) AMPs isolated from hemocyanin, (4) AMPs originated from the beta-subunit of hemoglobin (Hgbβ), and (5) cathelicidins, which are identified at the carboxyl-terminus of prepropeptides whose amino-terminuses are identical to a porcine serine protease known as cathelin (Zanetti et al., 1995; Smith et al., 2010; Sheshadri and Abraham 2012; Desriac et al., 2013).

Histones isolated AMPs

Histones are heat-resistant, highly alkaline, cationic proteins involved in chromatin structure. These proteins are also present in the cytosol and extracellular fluids (Hoeksema et al., 2016). Although the strong antimicrobial characteristics of histones have been identified for over 50 years (Hirsch, 1958), insufficient attention has been paid to this finding. In the late 1990s, Frohm et al. (1996) noted the role of histones in the antibacterial effects of wound blister fluid. Many investigators reported that histones from amphibians (Park et al., 1998 a) and teleost fish (Robinette et al., 1998; Patrzykat et al., 2001; Fernandes et al., 2002) epidermal mucus share great antibacterial activity. Histones have also been shown to possess high activity toward fish-parasitic dinoflagellates (Noga et al., 2001). Histones have also been reported in the epidermal exudates of various fish families, including Salmonidae, Siluridae, and Pleuronectidae (Smith et al., 2010). There is a great variety of histones, including core histones (lysine-rich histones [H2A, H2B] and arginine-rich histones [H3, H4]) that form the nucleosome and linker histones (H1 and H5) (Valero et al., 2013; Desriac et al., 2013; Masso-Silva and Diamond, 2014).

Core histones

Among the core histones, histone H2A from the epidermal mucus layer of rainbow trout (13.6 kDa) exhibits the most potent bactericidal activity, primarily targeting Gram-positive species (Fernandes et al., 2002). It has also shown a limited activity towards gram-negative bacterial species and the fungi *Saccharomyces cerevisiae* (Fernandes et al., 2002). The mode of action seems to create transitory ion channels in the cytoplasmic membrane of bacteria, however, when pure H2A comes into contact with a planar lipid bilayer, it can rupture the membrane (Fernandes et al., 2002). It is not only the whole H2A histone, but also the proteolytic cleavage portions of the amino terminus are antimicrobial agents (Table 1). For instance, parasin I (19-mer) from the catfish (*Ictalurus punctatus*) (Park et al., 1998 b), hipposin (51-mer) from Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo et al., 2003), abhisin from abalone (*Haliotis discus discus*) (De Zoysa et al., 2009), and buforins (39-residue) from Asian toads (Cho et al., 2009) are AMPs derived from proteolytic cleavage of H2A histone. In addition, intact H2A or parts generated from it have been found in crustaceans such as Pacific white shrimp (*Litopenaeus vannamei*) (Patat et al., 2004) and in bivalve mollusks such as scallops (*Chlamys farreri*) (Li et al., 2007). Although parasins are produced by skin-injured catfish, no peptides have been identified in healthy fish (Park et al., 1998 b). This explains the intervention of an inductive pathway in parasin I generation; it has been shown that the parasin I precursor is produced and stored in the cytoplasm (Cho et al., 2002 a). Cathepsin D mediates the release of H2A into catfish epidermal exudates (Cho et al., 2002 a) that it can be activated with another enzyme, matrix metalloproteinase 2 (MMP2) (Cho et al., 2002 b). Hipposin is a robust 51-residue AMP with a molecular mass of approximately 5.5 kDa originating from the surface-secreted mucus of Atlantic halibut. It displays sequence identity with parasin I (19-mer catfish AMP), buforin I (39-mer Asian toad AMP), and buforin II (a 21-mer part of buforin I) (Birkemo et al., 2004), implying that the three peptides exhibit the same antimicrobial strengths, even from different sources and at different sizes. This can be attributed to a general antimicrobial amino acid domain (a portion of the hipposin sequence), as all peptides are generated by proteolysis of the amino-terminus of histone H2A (Birkemo et al., 2004). This protein has antimicrobial effects (Fernandes et al., 2002) (Table 1). In addition, hipposin shares 98% homologoy with the amino-terminal fragment of histone H2A in rainbow trout (Fernandes et al., 2002).

These three peptides possess widespread and robust antibacterial activities towards both gram-positive and gram-negative species (Park et al., 1996, 1998 a; Birkemo et al., 2003, 2004). Moreover, parasin I was found to be lethal to numerous yeast species, such as *S. cerevisiae, Cryptococcus neoformans*, and *C. albicans*, but was not toxic to human red blood cells (Park et al., 1998 b). Structural studies demonstrated that parasin I adopts an amphipathic beta-strand organization, whereas hipposin appeared to adopt an amphipathic and a partly α-helical conformation (Park et al., 1998 b; Birkemo et al., 2004). The mechanism of action of hipposin is interesting considering that the complete hipposin sequence comprises buforin II and parasin I, which function through various antibacterial strategies. Parasin I destroys pathogens via cytoplasmic membrane permeabilization (Koo et al., 2008), whereas buforin II kills infectious species by binding to cellular DNA and RNA of the cells following entrance (Park et al., 1998 a). Using a modular study, Bustillo et al. (2014) explained that an existence of the amino-terminal region supported hipposin's membrane permeabilization. Hipposin, which lacks the amino-terminal region, loses its properties and acts like buforin II. Furthermore, they ascertained that the carboxyl terminus of hipposin, a cell-penetrating sequence, could easily enter the bacterial membrane (Bustillo et al., 2014). In addition, it has been revealed that basic amino acids (such as lysine or arginine) in the amino terminus are essential for the cytoplasmic membrane-binding property of parasin I, whereas the amphipathic organization is crucial for exerting antimicrobial activity through membrane permeabilization (Koo et al., 2008). In contrast, the acetylated amino-terminal group is not required for hipposin activ-

ity, as a synthetic peptide without this group possesses antimicrobial activity (Koo et al., 2008). Nevertheless, it is conceivable that the acetyl group has a protection role for natural peptides facing *in vivo* damage via proteases (Koo et al., 2008). Cutrona et al. (2015) revealed that enhancing the amount of arginine versus lysine residues in histone-derived AMPs improved their antimicrobial activity, regardless of their action strategies. A higher arginine proportion increases the membrane permeabilization of parasin I and promotes the translocation of buforin II (Cutrona et al., 2015). Overall, these findings provide additional evidence for designing novel antimicrobial molecules with various mechanisms of action.

Expression of histone-derived AMPs changes in response to several pathogenic species. For instance, H2A histones are broadly expressed in numerous tissues, including higher expression levels in the immune system tissues, such as the spleen and kidney. Following exposure of *Carassius aurutus* to *Aeromonas hydrophila*, the gene expression of H2A was upregulated in immune system tissues, such as the kidney, spleen, and liver, suggesting that H2A plays a critical role in the immune response to pathogen invasion (Kong et al., 2017).

The complete H2B protein is also an antimicrobial agent, which was first reported in mouse macrophages (Hiemstra et al., 1993) and consequently noted in the epidermal mucus of channel catfish (Robinette et al., 1998), surface exudates of the green tree frog, *Rhacophorus schlegelii* (Kawasaki et al., 2003), epidermal secretions from Atlantic cod (Bergsson et al., 2005), and hemocytes of Pacific white shrimp (Patat et al., 2004) (Table 1). Histone H2B possesses great activity towards the fish pathogenic microorganisms *Saprolegnia* and *A. hydrophilia* (Robinette et al., 1998). The H4 protein is another microbicidal factor from core histones and is an immune agent in human sebocyte exudates (Lee et al., 2009 a). Although histone H4 in shrimp hemocytes has antibacterial properties (Patat et al., 2004), records of its antimicrobial functions in other aquatic species are limited. Regarding histone H3, synthetic H3-like peptides have antibacterial properties (Tsao et al., 2009) and H3 exists in the skin mucus of hagfish (Subramanian et al., 2008 b).

Linker histones

Similar to the core histones, linkers have antimicrobial characteristics (Table 1). Histone H1 has antimicrobial properties and has been identified in various organisms, including humans (Kashima, 1991; Wang et al., 2002), mice (Hiemstra et al., 1993), marine species such as fish (Richards et al., 2001; Fernandes et al., 2004; Lüders et al., 2005) and shrimp (Patat et al., 2004). Histone H1 derived from coho salmon (*Oncorhynchus kisutch*) exhibits antimicrobial effects towards *E. coli* (Richards et al., 2001). Similar results were observed for a 26-mer histone H1 amino-terminal segment of Coho salmon (HSDF-1), which showed antibacterial activity against numerous pathogenic fish bacteria, including *Salmonella enteritica, Listonella anguillarum,* and *A. salmonicida* (Patrzykat et al., 2001). In winter flounder (*Pseudopleuronectes americanus*), histone expression is up-regulated following infection with pathogenic agents, which was synchronized with an increment in the anti-infective activities of fish mucus and serum (Patrzykat et al., 2001). These findings indicate the importance of AMPs in the systemic and the mucosal immune responses to external stimulants in fish. In addition, a 69-mer peptide with a molecular mass of 7.2 kDa derived from the H1 histone carboxyl-terminus of rainbow trout, named oncorhyncin II, is a highly potent antimicrobial agent, approximately 10 times stronger than cecropin P1, towards both gram-negative and gram-positive species, presumably via bacterial membrane destabilization (Fernandes et al., 2004). Oncorhyncin II exhibits hemolytic activity in trout red blood cells (Fernandes et al., 2004). In addition, a 30-mer, proline-rich histone H1 amino-terminus-acetylated peptide was isolated from the epidermal exudates of Atlantic salmon and named salmon antimicrobial peptide (SAMP H1) (Lüders et al., 2005). SAMP1 possesses an approximate molecular mass of 2.8 kDa and anti-infective characteristics towards both gram-positive and gram-negative species. It has been shown that proline residues are extremely important for their antimicrobial effect, whereas the amino-terminal acetyl region is not, considering that deacetylation did not decrease the activity (Lüders et al., 2005). In addition, for the first time, Lüders et al. (2005) accentuated the role of proline isomerization in AMPs' action, as activation through a peptidylproline isomerase is essential to obtain antibacterial activity against synthetic SAMP1 (Lüders et al., 2005). Hence, the H1 protein is not only an effective antibacterial factor as an intact molecule, but its amino- and carbo xyl-terminal fragments also exhibit antibacterial properties.

Intracellular proteins

In addition to core and linker histones, investigations of rainbow trout mucus have shown that additional intracellular proteins are powerful antibacterial agents against pathogenic species. One of the fish intracellular proteins is a 6–7 kDa amino terminus that is generated from H6, a non-histone chromosomal protein termed oncorhyncin III (Fernandes et al., 2003; Brunner et al., 2020). Oncorhyncin III is a salt-sensitive peptide, with hemolytic activity against rainbow trout red blood cells, and has highly potent activity towards a wide variety of bacteria based on its ability to destabilize planar lipid membranes (Fernandes et al., 2003; Brunner et al., 2020). Another intracellular protein with an antibacterial nature is a 6.6 kDa segment originating from the 40S ribosomal protein of rainbow trout surface exudates, named S30 (Fernandes and Smith, 2002; Brunner et al., 2020) (Table 1). Although this peptide hinders the growth of gram-negative and gram-positive bacteria, it displays the most potent bactericidal effects against gram-positive bacteria (Fernandes and Smith, 2002;

Brunner et al., 2020). Other proteins and peptides generated from the ribosome, namely L40 (6.4 kDa), L36A (12.3 kDa), and L35 (14.2 kDa), were derived from Atlantic cod skin mucus (Bergsson et al., 2005) (Table 1). The exceptional ability of core and linker histones and ribosome-isolated proteins to kill infectious agents indicates that these intracellular proteins play significant roles in the defense system.

Hemocyanin isolated-AMPs

Hemocyanin (Hc) is an ancient large copper-containing respiratory protein that evolved as one of the key molecules involved in oxygen transport in a broad spectrum of arthropodal groups such as chelicerates (spiders, scorpions, mites, and king crabs) and crustaceans (crabs, lobsters, crayfish, shrimp, and their allied species) (Zhao et al., 2021). Several Hc-derived AMPs have been reported in crustaceans; one group involves Hc subunits and their cleaved peptides, and the other group involves cleaved Hc fragments and their derivatives. In contrast to Hc-derived AMPs in horseshoe crabs, there is no isolated AMP in the mature Hc of crustaceans, thereby suggesting the multiple functions of Hc in crustaceans. Secondly, the cleavage of Hc-derived AMPs is proposed to involve the prophenoloxidase (proPO) cascade. It is assumed that the activation and modulation of the proPO system enable Hc-derived AMPs to acquire antimicrobial functions and bind to the cell wall of bacteria that are sensitive to cell walls (Li et al., 2023; Ji et al., 2024).

Hemoglobin β-isolated AMPs

Hemoglobin β (Hgbβ) possesses a tetramer structure, namely four subunits (two α and two β) with a well-known antibacterial activity (Hodson and Hirsch, 1958). In addition, some researchers have identified Hgβ-originated AMPs from red blood cells of humans and different animals including fish (Fernandes and Smith, 2004; Ivanov et al., 2005; Liepke et al., 2003; Mak et al., 2004; Parish et al., 2001; Seo et al., 2014). Ullal et al. (2008) reported the upregulation of three beta-subunits of hemoglobin (Hbβ) AMPs, including HbβP-1, HbβP-2, and HbβP-3, in the skin and gill epithelium of channel catfish during an outbreak of white spot parasitic disease caused by *Ichthyophthirius multifiliis*. It is conceivable that these AMPs are proteolytic outcomes of an Hgbβ, because HbβP-1, -2, and -3 are respectively homologous to the carboxyl-terminus (approximately 100%) of Hgbβ, carboxyl-terminus of HbβP-1, and the amino-terminus of Hgbβ (100%) (Ullal et al., 2008). It has also been determined that the heme part of Hgb does not play any role in antimicrobial action, and this activity originates from the protein section of Hgb, considering that elimination of the heme group did not cause any reduction in activity (Parish et al., 2001). HbβP-1 exhibits a slight overall cationic charge with a pI of almost 8.5, nevertheless, it seems to possess amphipathic organization with random-coiled amino and carboxyl-termini; thus it is believed that it fulfills its antimicrobial effects based on the carpet model, at least towards *I. multifillis* (Ullal et al., 2008). HbβP-1 carried a limited antibacterial activity towards some gram-negative species, including fish bacterial pathogens (*Vibrio alginolyticus* and *A. hydrophila*); however, it did not possess any effects towards tested gram-positive species, whereas displayed remarkably robust antiparasite activity towards three fish ectoparasites; *Tetrahymena pyriformis*, *I. multifiliis*, and *Amyloodinium ocellatum* (Dinoflagellata) with extremely selective, stage-specific activity to the feeding stage, trophozoite (trophont), of the last two (Ullal et al., 2008; Ullal and Noga, 2010). This specific activity of HbβP-1 suggests its potential to penetrate and damage the parasite, based on the varying lipid composition of the parasite's membrane in different developmental stages (Ullal and Noga, 2010). However, HbβP-1 was not lytic to channel catfish red blood cells and the G1B cell line showed a highly selective function towards the parasite membrane (Ullal et al., 2008; Ullal and Noga, 2010).

It has also been reported that the surface exudate of the stingray (*Potamotrygon henlei*) plays an antimicrobial role and acts as a pro-inflammatory mediator function (Conceição et al., 2012). For the first time, Conceição et al. (2012) identified a polypeptide, PcfHb, with great sequence homology to the Hgbβ in the stingray's skin-secreted mucus that shows antimicrobial effects. PcfHb has an approximate molecular mass of 17 kDa and is effective towards gram-negative (*E. coli*), gram-positive species (*Micrococcus luteus*), and yeast (*Candida tropicalis*) without being toxic to human red blood cells. In general, the results mentioned above show that the cleavage of proteins that are not directly linked to the immune system towards pathogenic microorganisms, is a universal strategy whereby aquatic organisms generate AMPs that are components of their innate immune system. Recently an antimicrobial protein belonging to the hemoglobin subunit beta-A-globin chain was isolated from the epidermal mucus extract in air-breathing fish (*Channa punctatus*) with maximum activity against *E. coli, S. aureus* as well as a fungal pathogen, *Helminthosporium gramineum* (Hussain and Ghosh Sachan, 2024).

Cathelicidins

Cathelicidins are cationic and amphipathic peptides that exert a wide range of effects associated with host defense responses. Cathelicidins are identified in the carboxyl terminus of prepropeptides, where the amino terminus is identical to that of a porcine serine protease known as cathelin (Zanetti et al., 1995). Fish cathelicidins were originally identified as antimicrobial molecules in the Atlantic hagfish (*Myxine glutinosa*) (Uzzell et al., 2003). After that, multiple cathelicidin genomic copies have been discovered in various fish species, whereas only one gene exists in humans (Katzenback, 2015), though they are not explained in this review because their relationship to skin mucus is unknown.

Table 1. Antimicrobial peptides isolated from fish epidermal mucus

Protein/peptide	Fragment	Source	Sequence	Activity	Reference
1	2	3	4	5	6
Pardaxin					
Pa1		*Pardachirus pavoninus*	GFFALIPKIISSPLFKTLLSAVGSALSSSGEQE		(Thompson et al., 1986)
Pa2		*P. pavoninus*	GFFALIPKIISSPIFKTLLSAVGSALSSSGGQE		(Thompson et al., 1986)
Pa3		*P. pavoninus*	GFFAFIPKIISSPLFKTLLSAVGSALSSSGEQE		(Thompson et al., 1986)
Pa4		*Pardachirus marmoratus*	GFFALIPKIISSPLFKTLLSAVGSALSSSGGQE	G+,G-/F/ V/C/W/H	(Primor and Tu, 1980)
Pa5		*P. marmoratus*	GFFALIPKIISSPLFKTLLSAVGSALSSSGDQE		(Adermann et al., 1998)
Pleurocidins					
WF1		*Pseudopleuronectes americanus*	GWGSFFKKAAHVGKHVGKAALTHYL	G+,G-/F/C/B	(Cole et al., 1997)
WF2		*P. americanus*	GWGSFFKKAAHVGKHVGKAALTHYLG	G+, G-/F	(Douglas et al., 2003 b)
WF1L		*P. americanus*	GKGRWLDRIGKAGGIIIGGALDHL	G+, G-/F	(Douglas et al., 2003 b)
WFX		*P. americanus*	RSTEDIIKSISGGGFLNAMNA	G+, G-/F	(Douglas et al., 2003 b)
WFY		*P. americanus*	FLGFLFHGIRHGIKAIHGMIH	G+, G-/F	(Douglas et al., 2003 b)
WF3		*P. americanus*	FLGALIKGAIHGGRFIHGMIQNH	G+, G-/F	(Douglas et al., 2001)
WF4		*P. americanus*	GWGSIFKHGRHAAKHIGHAAVNHYL	G+, G-/F	(Douglas et al., 2001)
Grammistins					
Group I					
Gs 1		*Grammistes sexlineatus*	LFGFLIKLIPSLFGALSNIGRNRNQ	G+, G-/ H	(Sugiyama et al., 2005)
Gs 2=Pp 4a		*G. sexlineatus*	LFGFLIPLLPHIIGAIPQVIGAIR-	G+, G-/ H	(Sugiyama et al., 2005)
Pp 4b		*P. punctata*	LFGFLIPLLPHLIGAIPQVIGAIR-		(Shiomi et al., 2000)
Pp A		*P. punctata*	LFGFLIPLLPHLIGAIPQVIGALR-		(Shiomi et al., 2000)
Group II					
Gs B		*G. sexlineatus*	-IGGIISFFKRLF	G+, G-/ H	(Sugiyama et al., 2005)
Pp 1=Gs D		*Pogonoperca punctata*	FIGGIISFFKRLF	G+, G-/ H	(Sugiyama et al., 2005)
Pp 2a		*P. punctata*	FIGGIISLIKKLF		(Shiomi et al., 2000)
Pp 2=Gs E		*P. punctata*	FIGGIISFIKKLF	G+, G-/ H	(Sugiyama et al., 2005)
Pp B		*P. punctata*	FIHHIVHAIGRLF		(Shiomi et al., 2000)
Group III					
Gs A		*G. sexlineatus*	WWRELLKKLAFTAAGHLGSVLAAKQSGW	G+, G-	(Sugiyama et al., 2005)
Gs C		*G. sexlineatus*	NWRKILGKIAKVAAGLLGSMLAGYQV–	G+, G-	(Sugiyama et al., 2005)
Pp 3		*P. punctate*	NWRKILGQIASVGAGLLGSLLAGYE—	G+, G-/ H	(Sugiyama et al., 2005)
Pp C		*P. punctata*	NWRKILGQIAKVGAGLLGSLLAGYES–		(Shiomi et al., 2000)

Table 1 – contd.

1	2	3	4	5	6
Myxinidin		*Myxine glutinosa*	GIHDILKYGKPS-NH2	G+,G-/F/ B/W	(Subramanian et al., 2009)
Pelteobagrin		*Pelteobagrus fulvidraco*	GKLNLFLSRLEILKLFVGAL	G+, G-/ F	(Su, 2011)
AJN-10		*Anguilla japonica*	GCPQTPRCTNYAEKGQCPPN	G+, G-/ F	(Liang et al., 2011)
H1 (Nucleus)	Whole protein (20.7 kDa)	*Salmo salar*	AEVAPAPAAAAPAKAPKKKAAAKPKKAGPSVGE-LIVKAVSASKERSGVSLAALK	G-	(Richards et al., 2001)
H1 (Nucleus)	SAMP H1	*S. salar*	AEVAPAPAAAAPAKAPKKKAAAKPKKAGPS	G+, G-	(Lüders et al., 2005)
H1 (Nucleus)	N-terminus (HSDF-1)	*Onchorhynchus kisutch*	AEVAPAPAAAAPAKAPKKKAAAKPKK		(Patrzykat et al., 2001)
H1 (Nucleus)	C-terminus (oncorhyncin II)	*O. mykiss*	KAVAAKKSPKKAKKPATPKKAAKSPKKVKKPAAA-AKKAAKSPKKATKAAKPKAAKPKAAKAKKAAP-KKK		(Fernandes et al., 2004)
H1 (Nucleus)	Oncorhyncin I	*O. mykiss*	SKGKKANKDVELARG		(Fernandes et al., 2004)
HistoneH2A (Nucleus)	Whole protein (13.5 kDa)	*Ictalurus punctatus*	AERVGAGAPVL	G+, G-/F	(Robinette et al., 1998)
HistoneH2A (Nucleus)	Whole protein (13.5 kDa)	*O. mykisss*	MSGRGKTGGKARAKAKTRSSRAGLQFPVGRVHR-LLRKGNYAERVGAGAPVLAAVLEYLTAEILELAG-NA…	G+/ H	(Fernandes et al., 2002)
HistoneH2A (Nucleus)	N-terminus (hipposin)	*Hippoglossus hippoglossus*	SGRGKTGGKARAKAKTRSSRAGLQFPVGRVHRLL-RKGNYAHRVGAGAPVYLKGRGKQGGKVRAKAK-TRSS	G+, G-	(Birkemo et al., 2003)
HistoneH2A (Nucleus)	N-terminus (parasin-1)	*Parasilurus asetus*	KGRGKQGGKVARAKAKTRSS	G+,G-/F	(Park et al., 1998 b)
HistoneH2B (Nucleus)	Whole protein (13.8 kDa)	*Gadus morhua*	PEVAKPAAKKGSKKAVSKVA.....SK…..	G-	(Ramos et al., 2012)
HistoneH2B (Nucleus)	Whole protein (15.5 kDa)	*I. punctatus*	PDPAKTAPKKGSKKAVTKXA	G-/ F	(Robinette et al., 1998)
HistoneH3 (Nucleus)	~1 kDa	*Myxine glutinosa*	Histone H3:MARTKQTARKSHGGKAPRTLLATKAARK SAPTTGGVKKPHRYRPGTVALREIRKYQK……..		(Zapata et al., 2006)
Protein H6	Whole protein (oncorhyncin III)	O. mykiss	PKRKSATKGDEPARRSARLSARPVPKPAAKPK-KAAAPKKAVKGKKAAENGDAKAEAKVQAAGD-GAG	G+, G-	(Fernandes et al., 2003)
40 Rsp (S30)	Whole protein (6.7 kDa)	*O. mykiss*	KVHGSLARAGK	G+	(Fernandes and Smith, 2002)
60 Rsp (L40)	Whole protein (6.4 kDa)	*G. morhua*	IIEPSLRMLAQKYNCDKMIXRXXYARLHPR…	G+, G-	(Bergsson et al., 2005)
60 Rsp (L36A)	Whole protein (12.3 kDa)	*G. morhua*	VNVPKTRRTYCKK…	G+, G-	(Bergsson et al., 2005)

Table 1 – contd.

1	2	3	4	5	6
60 Rsp (L35)	Whole protein (14.2 kDa)	*G. morhua*	AKIKARDLRGKKKEELLKQLDDLKNELSQL….AVKA…..	G+	(Bergsson et al., 2005)
Hemoglobin β (Hgbβ)	PcfHb	*Potamotrygon* cf. *henlei*	Hgbβ of red stingray (*Hemitrygon akajei*): VKLSEDQEHYIKGVWKDVDHKQITAKALERVFV….LVAEALSSNYH	G+, G-/F	(Qu et al., 2013)
β-defensin-like protein 1		*Cyprinus carpio*	PQSILVLLVLVVLALHCKENEAVSFPWSCASLSGVCRQGVCLPSELYFGPLGCGKGFLCCVSHF	G+, G-/F	(Shabir et al., 2022)

G+, G-: antibacterial activity against gram positive (G+) and gram negative (G-) bacteria; F: antifungal effect; V: antiviral effect; C: anticancer effect; B: antibiofilm effect; W: wound healing effect; H: hemolytic activity.

Cyclic cysteine-rich AMPs

The peptides of this group comprise conserved 6-cysteine residues, which constitute of intramolecular disulfide bridges and beta-strand structures (Katzenback, 2015). This group contains a remarkable variety of peptides including defensins and hepcidins or LEAP (formerly termed liver-expressed antimicrobial peptides) (Diamond et al., 2009).

Defensins

Defensin is the general title for cysteine-rich, cationic, and amphipathic antimicrobial peptides that form a homogenous AMP category and displays a secondary conformation composed of a common beta-strand core fixed via three disulfide bridges (triple-stranded beta-sheet proteins) (Zhu and Gao, 2013; Zhao and Lu 2014) which have been discovered in fungi, plants, invertebrates, and vertebrates including fish (Bulet et al., 2004; Aerts et al., 2008; Zhu and Gao, 2013). Although they have been extensively identified and described in fish, their mechanism of action on the skin mucus is not fully described. However, in a current study, β-defensin-like protein 1 with a molecular weight of 6908 Da was isolated and characterized from the crude epidermal mucus extract of common carp, *Cyprinus carpio*. This peptide demonstrated high antimicrobial activity against five bacterial and four fungal pathogens, with *Leclercia adecarboxylata*, *Enterobacter kobei* and *Candida glabrata* being the most susceptible (Shabir et al., 2022).

Hepcidins

Hepcidin is a blood-originating cysteine-rich peptide with a beta-strand secondary organization that plays a major role in iron homeostasis and direct antimicrobial activity against pathogenic species (Masso-Silva and Diamond, 2014; Luo et al., 2020; Barroso et al., 2021). Hepcidins were firstly isolated from humans (Krause et al., 2000; Park et al., 2001) and named liver-expressed antimicrobial peptide (LEAP-1); however, they have been discovered in several other vertebrates, including amphibians, reptiles, and fish, but have not been observed in birds or invertebrates (Hilton and Lambert, 2008; Barroso et al., 2021) and are not related to the skin mucus.

Other peptides with distinct conformation

Some cationic AMPs from aquatic animals are not included in any of the previously introduced classes, indicating a broad diversity of AMPs. AJN-10 and NK-lysin are included in this class (Valero et al., 2013). The reports have confirmed that these AMPs have no similarities with other AMPs in terms of the amino acid composition in the published documents to date.

AJN-10

AJN-10, with an approximate MW of 6 kDa, is a hydrophilic and heat-tolerant peptide that was isolated from epidermal exudates of the Japanese eel (*Anguilla japonica*) when infected with *A. hydrophila* (Liang et al.,

2011). This novel inducible biomolecule forms a unique disulfide bond and assumes beta-strand organization owing to the presence of three cysteine amino acid residues at its 20 amino-terminus end (Liang et al., 2011). Additionally, AJN-10 contains four proline residues that play important roles in its antimicrobial effects (Liang et al., 2011). Previous reports also identified a set of hydrophobic proteins with the MW of 43–67 kDa and a 45 kDa hydrophobic protein from surface extracts of Japanese eel, 27 and 31 kDa proteins from common carp (Lemaitre et al., 1996), 65 kDa protein from rainbow trout and 49 kDa protein from tench (*Tinca tinca*) (Ebran et al., 1999), and an approximate 400 kDa glycoprotein from the rabbitfish (*Siganus fuscescens*) (Nagashima et al., 2001) that exhibited a strong bactericidal activity against both gram-positive and gram-negative species through pore-forming action (Ebran et al., 1999, 2000). Recently, 126 proteins were differentially expressed in the skin mucus of grass carp (*Ctenopharyngodon idella*), most of which were immunological proteins that were significantly elevated in fish challenged with *A. hydrophila* infection (Ali et al., 2023 a).

NK-lysin

NK-lysin (NKL) is orthologous to human granulysin, which is principally produced by the granules of cytotoxic T lymphocytes (CTLs) and NK cells. These molecules structurally belong to the saposin-like protein (SAPLIPs) family, which displays lipid-binding activity and is, hence, able to dissipate through cytoplasmic membranes (Ruysschaert et al., 1998; Bruhn and Leippe, 1999; Bruhn et al., 2003). Teleost NKL is also expressed in turbot red blood cells and plays an essential role in their autophagic activity (Pereiro et al., 2017). Moreover, immunostaining tests have demonstrated the presence of NK lysins in the cluster of differentiation (CD) 2, 4, and 8 surfaces of T and NK cells (Andersson et al., 1995; Endsley et al., 2004). Fish NK lysins, similar to their mammalian counterparts, comprise six cysteine-conserved residues that form three intramolecular disulfide bonds (C1–C6, C2–C5, C3–C4) and assume a 3-D conformation displaying five amphipathic alpha-helixes folded into a single globular domain with a hydrophilic side (Andersson et al., 1995; Liepinsh et al., 1997; Wang et al., 2000; Hirono et al., 2007). NK-lysin has also been identified in the small intestine of pig (Andersson et al., 1995). In teleosts, they were derived from olive flounder (Andrä et al., 2004; Hirono et al., 2007), channel catfish (Wang et al., 2006), *Cynoglossus semilaevis* (Zhang et al., 2013), zebrafish (Pereiro et al., 2015), fugu, Atlantic salmon, large yellow croaker (*L .crocea*) (Zhou et al., 2016), Nile tilapia (Huang et al., 2018), turbot (Pereiro et al., 2017), common carp (Wang et al., 2018), gilt-head seabream and European bass (Valero et al., 2020 a). For the first time, EST-based NK-lysin-like transcripts from Atlantic salmon were detected and described that caused upregulated expression of *IL-1β* and *IL-8* genes in the head kidney leukocytes (Acosta et al., 2019).

Many reports have reflected that fish NK-lysins have a direct or indirect action towards pathogenic bacterial species (Wang et al., 2006, 2018; Hirono et al., 2007; Zhang et al., 2013, 2014; Cai et al., 2016; Zhou et al., 2016; Huang et al., 2018; Valero et al., 2020 b), fungi (Hirono et al., 2007), viruses (Díaz-Rosales et al., 2012; Zhang et al., 2013, 2014; Pereiro et al., 2017; Falco et al., 2019), and parasites (Lama et al., 2018). Furthermore, NK-lysins are lethal to a wide variety of tumor cells but are not lytic for red blood cells (Andersson et al., 1995). They also exhibit immunomodulatory capabilities (Acosta et al., 2019). Valero et al. (Valero et al., 2019) identified NK-lysin for the first time in the epidermal exudates of Atlantic salmon, which had higher levels of expression than in the serum, with bacteriostatic effects that reflect various defense approaches of AMPs, implying greater NK-lysin intervention in local immune responses (Valero et al., 2019).

Non-cationic or anionic AMPs (NCAMP or AAMP)

NCAMPs were initially identified in the early 1980s and have since been discovered as essential components of the non-specific immune system of vertebrates, invertebrates, and plants (Harris et al., 2009). These peptides have broad-spectrum effects on fungi, viruses, and gram-positive, and gram-negative bacterial species. NCAMPs can be constitutively expressed or induced, and post-translational modifications are necessary for their antimicrobial activity (Brogden et al., 2003; Harris et al., 2009). AAMPs show an overall negative charge and length ranging from -1 to -7 and 5 to 70 amino acids, respectively. Their anionic nature is attributed to the presence of aspartic and glutamic amino acid residues (Brogden, 1992; Rana et al., 2006). It has been proposed that NCAMPs evolved their functionality in response to the mechanisms of bacterial resistance against cationic AMPs and fulfill various mechanisms of action (Lai et al., 2002). However, the amphiphilic conformations of NCAMPs are different from those of alpha-helical peptides in several amphibians, and cyclic-cystine-knot organization has been reported in many plant peptides (Harris et al., 2009; Valero et al., 2020 b). Some AAMPs contain metal ions that form cationic salt bonds with negative charges to penetrate the pathogens' membrane (Brogden et al., 1996). This procedure results in the entry of these peptides into microbial cells, where they can target intracellular purposes (Brogden et al., 2003). Focusing on fish, three proteolysis-originated AAMPs from the scaly hairfin anchovy fish (*Setipinna taty*) (Song et al., 2012), SHβAP from Skipjack tuna (Seo et al., 2014), beta-defensin 1, 2 and 3 from Japanese halibut (Nam et al., 2010), hepcidin 1 and 2 from orange-spotted grouper (Zhou et al., 2011), piscidin 2 from Atlantic cod (Ruangsri et al., 2012), H2A protein from rainbow trout (Fernandes et al., 2002), and NK-lysin from yellow croaker (Zhou et al., 2016) have been reported.

Conclusion and future aspects

Although there are several excellent reviews on fish AMPs (Valero et al., 2013, 2020 b; Desriac et al., 2013; Masso-Silva and Diamond, 2014; Katzenback, 2015; Shabir et al., 2018; Wang et al., 2024), or fish mucosal immunology, and especially epidermal mucus (Esteban and Cerezuela, 2015; Beck and Peatman, 2015; Dash et al., 2018; Hedmon et al., 2018; Tiralongo et al., 2020), this review is the first comprehensive and deep study of the epidermal mucus-derived antimicrobial peptides produced by fish species, their classification, and the biological activities.

Fish epidermal mucus is an excellent source of AMPs, as it contains all the major classes of AMPs, including defensins, cathelicidins, hepcidins, histone-derived peptides, and a fish-specific class named piscidins. However, the structural uniqueness of mucus among fish species and its ability to be manipulated by endogenous and exogenous factors can be exploited by the pharmaceutical industry to find potent therapeutic agents. Antimicrobial peptides of epidermal mucus are unique molecules that are promising candidates to combat multidrug-resistant species as they could prevent and destroy the formation of biofilms and the formed ones as well as being equipped with a multi-hit mechanism. In addition, they may play multiple roles, such as antiviral, antitumor, antiparasitic, antifungal, antioxidant activities, and wound healing, as well as having anti-inflammatory and immunomodulatory properties. However, they are salt- and heat-tolerant AMPs and exhibit relatively weak cytotoxicity and hemolytic activities in mammalian cells, which has attracted a great interest in the scientific community. These features make fish epidermal mucus-derived AMPs potent therapeutic agents, not only for oncological treatments, immunogenic drugs, or immunological modulators in the future, but also as potential alternatives to conventional antibiotics or antivirals in aquaculture and the medical field, particularly in the context of the COVID-19 pandemic (Akhavan-Bahabadi, 2020; Sumon et al., 2021; Ali et al., 2022).

Author contributions

All authors contributed to the conceptualization, design and provided critical feedback. Mohammad Akhavan-Bahabadi performed the literature search and wrote the original draft of manuscript. Reviewing and editing the final version of the manuscript were done by Seyed Pezhman Hosseini Shekarabi and Ebru Yilmaz. All listed authors have approved the manuscript before submission, including the names and order of authors.

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