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Antimicrobial Peptides: A Potential Therapeutic Alternative for the Treatment of Fish Diseases

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Abstract: Fish losses from infectious diseases are a significant problem in aquaculture worldwide. However, peptide antibiotics show potent activity against a broad range of pathogens including fish pathogens. Therefore, the ability of antimicrobial peptides to protect fish against infections caused by fish pathogens clearly shows the potential for utilisation of antimicrobial peptides for the treatment of fish diseases. The strategy of overexpressing the antimicrobial peptide genes in transgenic fish may provide a method of decreasing fish disease problems in aquaculture.

Key Words: Antimicrobial peptides, cecropin, fish disease, aquaculture

Antimikrobiyal Peptidler: Balık Hastalıklarının Tedavisinde Potansiyel Bir Terapötik Alternatifi

Özet: Kültür balıkçılığında enfeksiyoz hastalıklar nedeniyle görülen balık kayıpları dünya çapında oldukça önemli bir problemdir. Antimikrobiyal peptidler olarak bilinen antibiyotikler balık patojenleri dahil pekçok patojen organizmayı etkili olarak öldürebilmektedir. Dolayısıyla patojenlerin oluşturdukları enfeksiyonlara karşı balıklar, antimikrobiyal peptidlerden yararlanılarak korunabilirler. Bu amaçla antimikrobiyal peptidleri kodlayan genlerin balıklara transferinin yapılması ile transgenik balık üretimi kültür balıkçılığı sırasında karşılaşılan balık hastalıklarının azaltılması açısından iyi bir strateji sağlayabilir.

Anahtar Sözcükler: Antimikrobiyal peptidler, cecropin, balık hastalıkları, kültür balıkçılığı

Introduction

All muticellular organisms are susceptible to infections because from a microbe's perspective their tissues are rich sources of nutrients. Therefore, multicellular organisms have developed defense systems to recognize invading microorganisms and eliminate them (1). The defense system of vertabrates consists of acquired and innate processes. The acquired process is characterized by combinatorial immune response capable of specifically recognizing and selectively eleminating foreign microorganisms and molecules. On the other hand, innate response refers to a basic resistance to infections encountered by an organism. Therefore, the innate defense mechanism blocks the entry of pathogens and eleminates pathogens in host body in a nonspecific fashion (2).

It has long been known that the innate defense system especially includes acute phase proteins such as C-reactive protein and its homologues. However, an important facet of this defense mechanism was revealed two decades ago by the discovery of cecropins, inducible antimicrobial peptides in the giant silk moth *Hyalophora cecropia*. In 1981, Boman and his associates reported that these widespread biochemical defense peptides act as antibiotics (1). After the discovery of cecropins from the giant silk moth, *Hyalophora cecropia*, antimicrobial peptides were able to be isolated from a number of organisms including mammals (3). Fish also possess antimicrobial peptides as part of their defense system. However, fish antimicrobial peptides are mainly located in the mucus layer indicating that they eliminate the pathogen bacteria before they pass the skin barrier. In intensive aquaculture conditions fish often lose their scales and get scars, which enable pathogen bacteria to pass the skin barrier easily (4,5). In addition, the culture conditions introduce stress factors that make fish susceptible to invasion by opportunistic bacterial pathogens. Despite the use of antibiotics and vaccination, an increasing number of outbreaks and the emergence of new pathogens such as new species of *Vibrio*, which were previously not known to cause infections, have been reported (6).

Fish losses from infectious diseases are a significant problem in aquaculture worldwide. The ability of antimicrobial peptides to protect fish against infection caused by fish pathogens has recently started to be investigated. The results of early investigations (7,8) revealed a potential for antimicrobial peptides to protect fish against infections. Therefore, the aim of this paper is to give introductory remarks about antimicrobial peptides that might be a good therapeutic alternative for the treatment of fish disease.

Discovery of Antimicrobial Peptides

Antimicrobial peptides were discovered by two independent lines of work: 1) studies on mechanisms by which mammalian phagocytic cells kill bacteria; and 2) studies on the mechanism by which organisms kill bacteria for their survival. In the late 1870s scientists were searching for an agent to kill microbes without causing unacceptable damage to the hosts. Ehrlich, who called this agent a "magic bullet", in the search for this agent started to work on mammalian granulocytes, and noted the different staining characteristics of these cell (9). In 1883, Metchnikov described the involvement of granulocytes in the phagocytosis of microbes (10). Two years later, Kanthack and Hardy discovered that the degranulation of granulocytes killed phagocytosed bacteria. In the following years Petterson found that aqueous extracts of pus from human emphysema had antimicrobial activity. Petterson and his coworkers desired to identify the compounds responsible for the antimicrobial activity. However, the techniques of the time were insufficient for further investigation of these antimicrobial agents (9). Approximately two decades later, Fleming's discovery of first lysozyme and then penicilin started a new era for the search of antimicrobial agents (10). Ten years after the discovery of penicilin Hotchkiss and Dubos isolated tyrocidine and gramicidin antimicrobial peptides from *Bacillus brevis*, but only gramicidin could be used for very limited applications because of the cytotoxic activity of these antimicrobial peptides on eukaryotic cells. In following decades other antimicrobial peptides were isolated, such as mellitin from bee venom, but they too were toxic and hemolytic (11). In 1969, Zeya and Spitznagel isolated five cationic antimicrobial proteins from rabbit polymorpho nuclear leukocytes that were not hemolytic, and found that cationic proteins permeate the bacterial cell because of their positive charge (12). In 1978, Weis and Elsbach reported the isolation of a protein, bacterial permeability inducing factor (BPI), from granule proteins of neutrophils of a chronic myelogenous leukemia patient. BPI had additional functions such as the neutralization of endotoxins besides its bactericidal activity (13). In the early 1980s, cecropins were discovered after a decade of work (11). Boman and his associates demonstrated that the hymolymph of silk moth pupae had no antimicrobial activity, but the introduction of bacterial debris induced potent antimicrobial activity in the hymolymph. Subsequently, they associated this activity with cecropins and some other antimicrobial peptides such as attacins and lectins. At first it was thought that these antimicrobial peptides were unique to insects, but later they were isolated from other animals including mammals revealing that these peptides were widely distributed in the animal kingdom and provide enormous survival benefits to the host (14). Because these peptides are very potent against bacteria, but have no toxic or hemolytic effect on host cells, and have a wide taxonomic distribution, their discovery led to the start of a new era in studies of animal antimicrobial peptides (3,15,16)

Classification of Antimicrobial Peptides

As was mentioned earlier, antimicrobial peptides were discovered as a result of two independent lines of work: first, studies on how mammalian phagocytic cells kill bacteria, and second, on how organisms kill bacteria. Therefore, in the past the origin of antimicrobial peptides was the basis for classification because this type of classification helped to make connections between the function of the antimicrobial peptides originated from a similar group of animals and aspects of the living conditions of these animals. However the later discovery of a large number of peptides from many different animal species and the possession of a group of antimicrobial peptides, such as cecropins, by distantly related animal groups caused this type of classification to become futile. Today, a grouping approach based on the chemical and biochemical characteristics of peptides is preferred. The present grouping combines both sequence homologies, three-dimensional structures and functional similarities. According to this clasification antimicrobial peptides can be reviewed in five groups (3).

1) Linear, mostly helical peptides without cystine residue, with or without a hinge region. Cecropins (Fig. 1), magainins and bombinins are in this group (3).

2) Linear peptides without cyteine residue and with a high portion of certain residues. Attacins and diptericins from this group contain glycine-rich and proline-rich domains (17).

3) Antimicrobial peptides with one disulfite bond that form a loop structure with tail(s). Bactenecin, brevinins and esculentin are examples of this group (3).

4) Antimicrobial peptides with two or more disulfite bonds giving mainly or only β-sheet structure. Defensins are well known representatives of this group (18).

5) Antimicrobial peptides with other known functions. These are the molecules derived from larger peptides with other known functions by postranslational processing or alternative splicing. GIP(7-42), derived from gastric inhibitory polypeptide, and DBI(32-86), derived from diazepam-binding inhibitor, are in this group (3).

Antimicrobial Peptides Isolated from Fish

Fish are one of the organisms that have managed to survive in a milieu of pathogenic organisms (19). The primary interference of fish with their environment happens through a mucus layer that covers their entire body. Research has demonstrated that the mucus layer is composed of biochemically diverse secretions from epidermal goblet cells and epithelial cells (16). It was reported that epithelial tissues produce antimicrobial molecules which serve as the first line of a host's defense against microbial invasion in a variety of vertebrates including humans (18).

Figure 1. Schematic representation of structure of cecropin A. Taken from Holak et al. (1998). The position of charged (+/-) and polar residues are indicated.

The protective role of the surface mucus layer in fish was investigated by Austin and McIntosh (1998) in rainbow trout and they demonstrated that antimicrobial compounds from this layer predominantly inhibited the growth of *Aeromonas hydrophila* (19). Similarly, Fouz et al. in 1990 reported that the mucus of turbot had antibacterial activity against different pathogenic bacteria (5). Unfortunately, the results from these studies were obtained using the crude mucus layer resuspended in water and therefore included a mixture of compounds. However, the encouraging results from the above studies done using crude mucus layer extract prompted researchers to identify the compounds in the mucosal barrier. Bacteriolytic ubiquitous enzymes and lysozymes were first noticed for their antibacterial activity in fish but, later it was shown that they promote phagocytosis as an opsonin, or by directly activating polymorphonuclear leukocytes and macrophages. In fish, lysozyme is distributed mainly in tissues rich in leucocytes, such as the head kidney, and at sites where the risk of bacterial invasion is high, such as the skin, gills, alimentary tract, and in the eggs (20).

A 33-amino-acid pore forming peptide pardaxin, was one of the early discovered antimicrobial peptides. Lazarovici et al. (1986) isolated pardaxin from the secretions of the Red Sea Moses sole fish, *Pardachirus marmoratus* (21). It has been reported that this peptide has a helix-hinge-helix structure similar to cecropin and mellitin, and it is an excitatory toxin that possesses high antibacterial activity and low hemolytic activity towards human red blood cells compared with mellitin (22).

In 1996, two novel antibacterial proteins, 31 kDa and 27 kDa, were isolated from the skin mucosa of the carp *Cyprinus carpio* by Lemaitre et al. When the sequence of one of these hydrophobic proteins, 31-kDa, was compared with sequences in protein data banks, no similarities to other proteins were found. The 27-kDa protein was blocked at the N-terminus and thus its sequence was undetermined. These proteins had strong bactericidal activity against fish pathogens, but their hemolytic activity was not investigated (23).

Cole et al., in 1997 reported a novel 25-amino acid residue linear antimicrobial peptide pleurocidin, found in the skin mucous secretions of the winter flounder, *Pleuronectes americanus*. Pleurocidin had a high degree of homology with dermaseptin from hylid frog and ceratotoxin from Mediterranean fruit fly (24).

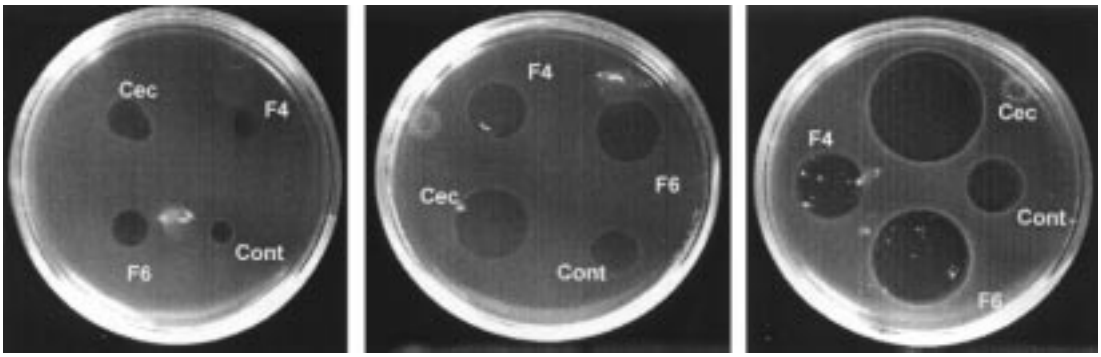

Figure 2. Inhibition zone assays demonstrating the bactericidal activity of silk moth cecropin B produced in the fish cell clones carrying silk moth cecropin transgenes. Taken from Sarmaşık (2000). A, *Aeromonas hydrophila*. B, Pseudomonas fluorescens. C, *Vibrio anguillarum*. Cec: synthetic cecropin antibiotic (Sigma), Cont: cell media from control cells, F4 and F6: cell media containing cecropins produced in F4 and F6 clones of Chinook salmon embryo cells.

Park et al., in the same year, isolated a novel strongly basic antimicrobial peptide, 21-amino-acid, from the loach (mudfish), *Misgurnus anguillicaudatus*, and called it misgurin. Misgurin amino acid sequence did not show any homology with other known antimicrobial peptides, but because of its structure it was grouped with cecropin, magainins, and dermaseptins. Misgurin showed strong antimicrobial activty against various microorganisms and slight hemolytic activity (25).

Three antimicrobial proteins were isolated from catfish skin in 1998. The molecular masses of the proteins were 15.5, 15.5, and 30 kDa. Amino acid composition and amino acid sequence data suggested that these proteins are closely related to histone H2B proteins. These H2B-like proteins inhibited the growth of *A. hydrophila* and *Saprolegnia* spp (26).

Zasloff's group isolated a steroid antimicrobial compound, squalamine, from the stomach as well as from various other organs of the dog shark, *Squalus acanthias*. Although a handful of antimicrobial steroids have been isolated from plants, squalamine is the first antimicrobial steroid isolated from an animal (27).

Mechanism of Action for Antimicrobial Peptides

The precise mechanism of action for antimicrobial peptides is yet to be explained. Nevertheless, studies show that prokaryotic membranes are recognized as targets by many antimicrobial peptides. Therefore, a number of models have been proposed to understand the mechanism of action of these peptides. According to one of the models, the mechanism involves the following steps: 1) electrostatic contact between a negatively charged membrane and positively charged antimicrobial peptide, 2) conformation of helical structure and insertion of the peptide into the membrane, and 3) aggregation of several helices to form a pore. It was reported that a micromolar range of antimicrobial peptides sufficient to form a monolayer around a target cell was required for the lysis of bacteria and four or more peptides are required to aggregate and form pores, 5-40 Å in diameter, large enough to kill a target cell. However, it was thought that an organism may be killed in different ways by different peptides, even if they are in the same structural class, or a peptide may operate by different mechanisms on different organisms (28).

Antimicrobial peptides are preferentially more selective to the procaryotic cell membrane. This might be because prokaryotic cell membranes are more anionic, and prokaryotic cell membranes do not have cholesterol. Studies showed that the presence of cholesterol in the artificial membranes significantly reduced the lytic activity of antimicrobial peptides. Research also demonstrated that besides the antibacterial activity antimicrobial peptides also possesses antitumor, antiviral and antiparasitical activity (17).

Since antimicrobial peptides posses a different mechanism of action than other antibiotics, they can be still active on some bacteria which develope resistance to common antibiotics. However, it was thought that bacteria that produce proteolytic enzymes may gain resistance to some antimicrobial peptides such as

cecropins, which can be cleaved by proteases. However, other studies showed that many bacteria with proteolytic enzymes such as *Pseudomonas* were still susceptible to cecropins (16).

Future Role of Antimicrobial Peptides as Therapeutic Agents

Among all multicellular organisms encountering microbe-laden environments, insects show considerable success in eliminating primary infections caused by intruder organisms. This is because they possess potent antimicrobial peptides as an important part of their innate defense system. The main advantage of antimicrobial peptides for innate defense is that they are small molecules and can be synthesized in a matter of hours, unlike components of adaptive immune response, which take days, and can eleminate two or more intruders at the same time without requiring specific recognition for each foreign invader (3).

As was mentioned earlier fish too possess antimicrobial peptides. In nature, where there are fewer stress factors present, their native antimicrobial peptides may be sufficient to protect fish against infections. However, in aquaculture facilities fish not only have to live in a plethora of microbes, but also encounter stress and physical injuries caused by other fish or the environment itself. These conditions set up the optimal circumstances for pathogens to prey on the susceptible host (6,19).

Currently strategies to control fish diseases consist of prophylaxis, antibiotic or chemical treatment, when feasible, and eradication of an infected group of fish. The application of vaccine-based immunization strategies is very limited; there are only a handful of approved antibiotic available, the number of resistant bacteria to existing antibiotics is increasing, the use of chemicals is subject to increasing restriction because of their potential harmful impact on the environment, and eradication of an infected fish group is feckless often because by the time disease is diagnosed most of the fish are infected. Thus, the control of fish diseases in most cases is limited to hygiene. It is obvious that hygiene alone is not sufficient. Therefore, urgent precautions have to be taken to control fish disease (6).

The development of natural disease resistance may be the most effective method to control fish disease. To date conventional techniques such as selection and hybridization have been used to produce a number of resistant fish for broodstocks using the existent resistant individuals in heterogeneous fish stocks. However, conventional techniques are restricted to within species or closely related species, and do not allow for the employment of potentially effective resistance genes possessed by other organisms. On the other hand, modern approaches like recombinant DNA technology enable the transfer of genes from diverse sources such as insects to improve the natural resistance of fish to infections in a directed fashion (29).

Because previous *in vitro* studies demonstrated that insect and porcine cecropin genes expressed in *E. coli* were effective at killing a wide range of bacteria, enveloped viruses, unicellular parasites and tumor cells (3), recent *in vitro* studies have been conducted to demonstrate that fish cells carrying cecropin transgene constructs are able to produce bactericidally active cecropins (8). The inhibition zone assays done using the cecropins synthesized in fish cells revealed the bactericidal activity against fish bacterial pathogens *Aeromonas hydrophila, Pseudomonas fluorescens,* and *Vibrio anguillarum* (Fig. 2).

The impressive results obtained from the *in vitro* experiments prompted researchers to test the activity of these antimicrobial peptides in *in vivo* systems. Results obtained from one of the *in vivo* studies showed that a cecropin B homolog transgene could augment the resistance of transgenic tobaco plants against bacterial infections (30). As a step towards the enhancement of bacterial resistance of fish, in another *in vivo* study Japanese medaka was used as a model fish system and transgenic fish carrying a cecropin gene transgene construct were produced. The transgenic fish were then challenged with two fish pathogen bacteria, *P. fluorescens* and *V. anguillarum*, to determine whether cecropin had enhanced the resistance of transgenic medaka. The results of this study showed that transgenic medaka were more resistant to pathogen bacteria in comparison to control fish (8). Hancock et al. employed a different strategy in their most recent study where antimicrobial peptides were delivered continuously using miniosmotic pumps placed in the peritoneal cavity of coho salmons. Then these treated fish received intraperitoneal injections of *V. anguillarum*. Fish receiving antimicrobial peptides through miniosmotic pumps survived longer and

had significantly lower accumulated mortalities than the control groups that did not receive antimicrobial peptides (7).

Besides revealing the elevated level of host protection against pathogens, *in vivo* studies proved that insect borne diseases such as malaria can be prevented using insects carrying symbiotic bacteria transformed with an antimicrobial peptide gene, and transgenic mice can be used to produce tracheal antimicrobial peptides, potential antibiotics for the treatment of cystic fibrosis. Currently, antimicrobial peptides are in trials for their use in clinics for the treatment of skin infections associated with burns, diabetic wounds, and eye infections (3,11). Evidently, the genes encoding these potent antimicrobial peptides represent good candidates for the genetic improvement of fish stocks to react to bacterial diseases.

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