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Short communication

Piscidin: Antimicrobial peptide of rock bream, *Oplegnathus fasciatus*

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ABSTRACT

The piscidin family consists of antimicrobial peptides (AMPs) that are mainly found in fish and are crucial effectors of fish innate immune responses. The piscidin family typically has broad-spectrum antimicrobial activity and can modulate immune responses. In this study, we cloned rock bream piscidin (Rbpisc) and investigated its gene expression and biological activity (including antimicrobial and cytotoxic activities). The coding region of Rbpisc consisted of 213 base pairs (bp) encoding 70 amino acid residues. The tertiary structure predicted for Rbpisc includes an amphipathic helix-loop-helix structure. The Rbpisc gene was highly expressed in the gills of healthy fish. The gene expression of Rbpisc increased in the gills after pathogen infection, while the expression was down-regulated in other tissues. A synthetic peptide based on the AMP 12 domain amino acid sequence of Rbpisc appeared to have broad-spectrum antimicrobial activity against various bacteria. However, the synthetic peptide exhibited weak haemolytic activity against fish erythrocytes. These results suggest that Rbpisc might play an important role in the innate immune responses of rock bream.

1. Introduction

To prevent many infectious diseases in fish farms caused by bacteria, fungi and viruses, large amounts of antibiotics are routinely used. However, wrong or improper use of antimicrobial agents has increased the number of strains resistant to these agents, which can potentially impact the treatment of fish diseases or the environment in the fish farms [1]. Aside from concerns about antibiotic resistance, there is a need for new approaches to combat serious infectious diseases [2].

Antimicrobial peptides (AMPs) found in virtually all life forms as nature's antibiotics [3,4] are considered a critical first line of defence against many microbial pathogens [1,5–7] They have shown promise as the basis for a new generation of antibiotics [8]. Generally, AMPs are present short (12–50 residues) cationic amphipathic peptides, typically having broad-spectrum antimicrobial activity against many pathogens including multidrug-resistant bacteria, and they can modulate the immune response through various mechanisms [9,10].

AMPs defend the host against pathogens by direct antimicrobial action [11]. The amphipathic structure of the AMPs has been proposed to be crucial for antimicrobial activity [12]. The expressed peptide contains hydrophobic and hydrophilic residues and a positively charged hydrophobic face that interacts electrostatically with the anionic bacterial membrane [13–15]. The hydrophobic face of the peptide interacts with hydrophobic phospholipids and forms a pore, leading to extermination of pathogens [16].

Piscidin, an AMP isolated from fish, belongs to the same family as pleurocidin, moronecidin, chrysophisin, dicentracin, epinecidin and myxindin [17]. Since piscidin was first isolated from the mast cells of commercially cultured hybrid striped bass (white bass, *Morone chrysops*, female, ×striped bass, *Morone saxatilis*, male) [17], it has been characterized in various teleost species including Atlantic cod (*Gadus morhua*) [18], red sea bream (*Chrysophrys major*) [19], European bass (*Dicentrarchus labrax*) [20], grouper (*Epinephelus coioides*) [21], rainbow trout (*Oncorhynchus mykiss*) [22,23], striped bass (*M. saxatilis*) [24–26] and winter flounder (*Pleuronectes americanus*) [27]. The piscidin family of peptides also

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has an alpha-helical structure, low molecular weight, broad-spectrum antimicrobial activity and a cationic charge at physiological pH [17,28–30]. These peptides are present in most common cell types and in various tissues. Especially, they are found at mucosal surfaces such as the skin, gills and alimentary canal [31,32], and the expression of these genes is able to induced after pathogens infection [28,33].

Rock bream (*Oplegnathus fasciatus*) is considered to be a commercially important marine fish in Asia. However, many serious infectious diseases such as *Streptococcus* and red sea bream irido virus (RSIV) are widespread in rock bream aquaculture, causing substantial losses for the fish aquaculture industry. In this study, we identified a piscidin gene (Rbpisc) from the gill of rock bream and described its molecular characterization. Expression levels of Rbpisc in various tissues of healthy and pathogen-infected fish were examined. Furthermore, to confirm the possible use of Rbpisc as an antibiotic in aquaculture, its antimicrobial activity and cytotoxicity were investigated using a synthesised mature peptide.

2. Materials and methods

2.1. Molecular characterization of Rbpisc cDNA

Full-length Rbpisc cDNA sequence was obtained from expressed sequence tag (EST) analysis of gill from rock bream stimulated with lipopolysaccharide (LPS) (GenBank accession number of Rbpisc is BAM99884). Nucleotide and deduced amino acid sequences were determined using GENETYX ver. 8.0 (SDC Software Development, Tokyo, Japan). Molecular weight and isoelectric points (p*I*) of Rbpisc were predicted using the ProtParam tool on the ExPASy Proteomics Server (http://web.expasy.org/protparam/). Positions of the signal peptide and characteristic domains were identified with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Multiple sequence alignments were analysed using ClustalW (http://www.genome.jp/tools/clustalw/) with other piscidin family amino acid sequences. A phylogenetic tree analysis based on the AMP amino acid sequence in rock bream and other species was constructed using the neighbour-joining algorithm in MEGA ver. 4.0 [34]. Bootstrap sampling was performed with 2000 replicates. The Shiffer–Edmundson helical wheel diagrams and the hydrophobicity and the hydrophobic moment were predicted using HeliQuest from http://expasy.org/tools [35]. Tertiary structure prediction was performed using the I-Tasser server (http://zhang.bioinformatics.ku.edu/I-TASSER/about.html) [36,37].

2.2. Fish

The mean weight and body length of the fish used in this study were 68.5 ± 10 g and 14.3 ± 1 cm, respectively, and the fish were supplied by the Gyeongsangnam-do Fisheries Resources Research Institute (Tongyeong, Republic of Korea). Fish were maintained at 20–23 °C in aerated seawater and fed an extruded pellet to satiety once a day. All animal experiments were performed in accordance with the ethical guidelines of Gyeongsang National University.

2.3. Gene expression analysis of Rbpisc in different tissues of healthy fish

To evaluate gene expression of Rbpisc in rock bream, the fish were killed using an overdose of benzocaine (Sigma, St. Louis, MO, USA). Peripheral blood leukocytes (PBLs) and red blood cells (RBCs) were isolated using Percoll density gradients (Sigma, St. Louis, MO, USA) as described previously [38], and various tissues including the head kidney, trunk kidney, spleen, liver, intestine, gill, muscle, heart, skin and stomach were isolated from three healthy rock breams. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (RNase-free) (Takara, Shiga, Japan) to digest DNA molecules. The quality of purified total RNA was quantified using NanoVue (GE Healthcare Life Sciences, Uppsala, Sweden). First-strand cDNA synthesis was carried out using a first-strand cDNA synthesis kit (Takara, Shiga, Japan) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with SYBR Green Master Mix (Takara, Shiga, Japan) following the manufacturer's protocol. qRT-PCR was carried out with cDNA templates from each tissue and specific primer sets. The forward and revers primers for Rbpisc (5′-GAA CCT GGA GAG GGC TTT TT-3′ and 5′-CCT GGG TTG TAA TCG ACT GA-3′, respectively) were designed using the Primer3 v. 0.4.0 program (http://bioinfo.ut.ee/primer3-0.4.0/). Amplification was performed by initial denaturation at 50 °C for 4 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 20 s and 60 °C for 1 min, with a final dissociation at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The relative expression of Rbpisc gene was determined using the rock bream EF-1α gene as an internal reference by the comparative Ct ($2^{-\Delta\Delta CT}$) method [39] according to the Thermal Cycler DICE Real-Time System (Takara). All data are reported as the level of Rbpisc mRNA relative to EF-1α mRNA and expressed as the mean ± standard deviation (SD). Significant differences in gene expression among tissues were determined by analysis of variance (ANOVA).

2.4. Gene expression analysis of Rbpisc after pathogen infection

To analyse the immune response of Rbpisc against different pathogens, healthy rock bream were challenged with *Edwardsiella tarda*, *Streptococcus iniae* or red seabream iridovirus (RSIV) according to our previous study with minor modification [38]; they were adjusted to 1.5×10^5, 1.5×10^5 cells/fish and 1×10^4 copies/fish in phosphate buffered saline (PBS), respectively, and intraperitoneally injected. Used pathogens have been isolated from dead fish and analysed via necropsy and the PCR method. Control fish were injected with PBS alone. Injected fish were kept in aerated seawater at 23–26 °C and were not fed. Kidney, spleen and gill were taken from three fish at 1, 3, 5 and 7 days post-injection. Total RNA extraction and cDNA synthesis for qRT-PCR were conducted as described above. Additionally, qRT-PCR was performed with SYBR Green Master Mix (Takara, Shiga, Japan), and induced Rbpisc gene was expressed relative to EF-1α gene as the mean ± SD. Significant differences in gene expression levels between the pathogen-infected and PBS-injected (control) groups were determined by ANOVA.

2.5. Synthetic peptide

The mature peptide of Rbpisc was commercially synthesised by GL biochem (Shanghai, China) based on the amino acid sequence of the AMP 12 domain excepted the signal peptide region (GEGFLGMLLHGVGHAIHGLIHGK). Synthetic peptide was purified to 95% with HPLC. It was dissolved in 90% acetonitrile (2 mg/ml) and stored at 4 °C until use.

2.6. Antimicrobial activity assay

The gram-negative and -positive bacterial strains used in this study are listed in Table 1. Almost all bacterial strains (except *Escherichia coli*) were successfully cultured on brain heart infusion agar (BHIA) plates. *E. coli* was maintained on Luria-Bertani (LB) agar plates. The minimal inhibitory concentration (MIC) was measured by detecting the lowest concentration of an antimicrobial agent at which no bacterial growth occurred. The MIC of a peptide was