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Expression analysis and biological activity of moronecidin from rock bream, *Oplegnathus fasciatus*

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ABSTRACT

The piscidin-family, one of antimicrobial peptides (AMPs) mainly distributed in fish, is crucial effectors of fish innate immune response. Piscidin-family typically has broad-spectrum antimicrobial activity and the ability to modulate the immune response. In this study, we identified moronecidin (Rbmoro) included in piscidin-family from rock bream and investigated its gene expression using quantitative real-time PCR and biological activity (including antimicrobial and cytotoxic activity). The coding region of Rbmoro was 204 bp encoding 67 amino acid residues. Tertiary structure prediction of Rbmoro showed an amphipathic α-helical structure. Rbmoro gene was widely expressed in different tissues of healthy fish. Additionally, Rbmoro gene expression was induced in all tested tissues after infection with *Edwardsiella tarda*, *Streptococcus iniae* and red seabream iridovirus. We synthesized mature peptide of Rbmoro based on amino acid sequence of its AMP 12 domain, and the synthetic peptide appeared broad-spectrum antimicrobial activity to various bacteria. However, the synthetic peptide has weak haemolytic activity against fish erythrocytes. These results suggest that Rbmoro might play an important role in innate immune response of rock bream.

1. Introduction

Rock bream (*Oplegnathus fasciatus*) is considered commercially important marine fish. However, in recent years, rock bream has been afflicted by serious infectious disease, which causes huge losses in the fish culture industry. Most of these diseases are caused by bacteria, fungi and viruses. Large amounts of antibiotics have been used in the fish farms to prevent these outbreaks, but the wrong or improper use of antimicrobial agents in aquaculture has increased the frequency of strains resistant to these agents. Potentially these resistant strains can have an impact on the therapy of fish diseases or the fish farms environment [1]. Thus, there have been many recent attempts to fine effective replacements for antibiotic use [2].

Teleost rely significantly on their innate immune systems to combat the constant threat of infection in the aquatic environment [3]. Antimicrobial peptides (AMPs) considered as a new generation of antibiotics [4] are crucial effectors of innate immunity response and present in virtually all life forms as nature's antibiotics [5,6].

Generally, AMPs are present as short (12–50 residues) and cationic amphipathic peptides, and typically having broad-spectrum antimicrobial activity and the modulate the immune response through a variety of mechanism [7,8]. The piscidin-family, one of AMPs mainly distributed in fish, shares the properties of an alpha-helical structure, low molecular weight, broad-spectrum antimicrobial activity and cationic charge at physiological pH, and having potent antimicrobial activity against virus, bacteria, fungi and parasites [9–12].

The piscidin-family includes a variety AMPs such as pleurocidin, moronecidin, chrysophisin, dicentracin, epinecidin and myxindin, and highly conserved histidine-rich, phenylalanine-rich N-terminus and a more variable C-turminus [12]. Since it was firstly isolated from mast cells of the commercially cultured hybrid striped bass (white bass, *Morone chrysops*, female, × striped bass, *Morone*

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saxatilis, male) [12], it has been characterized from various species of teleost including Atlantic cod (*Gadus morhua*) [13,14], red sea bream (*Chrysophrys major*) [15], European bass (*Dicentrarchus labrax*) [16], grouper (*Epinephelus coioides*) [17], rainbow trout (*Oncorhynchus mykiss*) [18,19], striped bass (*Morone saxatilis*) [20–22] and winter flounder (*Pleuronectes americanus*) [23]. The piscidin-family presents in the most common cell types and various tissues, particularly mucosal surfaces such as skin, gill and alimentary [24,25], and expression of these genes is inducible by pathogens infection [11,26].

In this study, we identified moronecidin (Rbmoro) from the rock bream, and described the molecular characterization of Rbmoro. The expression level of Rbmoro in different tissues of health fish and the gene regulation after *Edwardsiella tarda*, *Streptociccus iniae* and red seabream iridovirus (RSIV) infection was examined. Furthermore, we investigated antimicrobial activity and cytotoxity of Rbmoro using synthesized mature peptide.

2. Materials and methods

2.1. Molecular characterization of Rbmoro cDNA

Full length of Rbmoro cDNA sequence was obtained from expressed sequence tags (ESTs) analysis of liver from rock bream stimulated with lipopolysaccharide (LPS) (GenBank accession number of Rbmoro is BAM99885). 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*) 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/). The phylogeny was inferred using the Mega 4 program and distance analysis using the neighbour-joining method [27]. Bootstrap sampling was performed with 2000 replicates. The Shiffer–Edmundson helical wheel diagrams and the determination of the hydrophobicity and the hydrophobic moment were predicted by using the HeliQuest from http://expasy.org/tools [28]. Tertiary structure prediction was performed using the I-Tasser server (http://zhang.bioinformatics.ku.edu/I-TASSER/about.html) [29,30].

2.2. Gene expression analysis of Rbmoro in different tissues of healthy fish

To evaluate gene expression of Rbmoro, various tissues including the head kidney, trunk kidney, spleen, liver, intestine, gill, muscle, heart, skin and stomach were isolated from three healthy rock breams. The weight of fish used in this study is 68.5 ± 10 g and body length is 14.3 ± 1 cm. Peripheral blood leukocytes (PBLs) and the red blood cells (RBCs) were isolated using Percoll density gradients (Sigma–Aldrich, St. Louis, MO, USA) as described previously [31]. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 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 PCR was performed with SYBR Green Master Mix (Takara, Shiga, Japan) following the manufacturer's protocol. Quantitative real-time PCR was carried out with cDNA templates of each tissue and specific primer sets of the Rbmoro gene (Table 1). 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 Rbmoro gene was determined by the $2^{-\Delta\Delta CT}$ method [32] using EF-1α expression as a reference. All data were reported as the level of Rbmoro 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).

Table 1
PCR primers used in this study.

Target	Usage	Primer sequence (5′–3′)
Rbmoro	Real time F	GTCGTCCTCATGGCTGAAC
	Real time R	CGTCCCCCAGTGATAAGTCT
EF-1α	Real time F	CCCCTGCAGGACGTCTACAA
	Real time R	AACACGACCGACGGGTACA

2.3. Gene expression analysis of Rbmoro after pathogens infection

To analyse the immune response of Rbmoro against different pathogens, healthy rock bream were challenged by intraperitoneal injection with *E. tarda*, *S. iniae* or RSIV, which 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 were kept in seawater at 23–26 °C. Control fish were injected with PBS alone. Kidney, spleen and gill were taken from three fish at 1, 3, 5 and 7 day post-infection. Total RNA extraction, cDNA synthesis and real-time PCR were conducted as described above. The significant differences of gene expression levels between the pathogens-infected and PBS-infected (control) groups were determined by ANOVA.

2.4. Synthetic peptide

The mature peptide of Rbmoro was commercially synthesized (GL biochem, Shanghai, China) based on amino acid sequence of Antimicrobial peptide (AMP) 12 domain. Synthetic peptide was purified with HPLC to 95%. It was dissolved in 90% acetonitrile (ACN) and stored at 4 °C until using.

2.5. Antimicrobial activity assay

The Gram-negative and positive bacterial strains used in this study are listed on Table 2. The minimal inhibitory concentration (MIC) was measured as detecting the lowest concentration of an antimicrobial agent at which no bacterial growth. The MICs of peptide were determined by a broth microdilution method [33]. Each bacteria strain was maintained on brain heart infusion agar (BHIA) plate. *Escherichia coli* were maintained on Luria–Bertani (LB) agar plate. For liquid culture, all bacteria strains expect for *E. coli* were grown in BHI broth (BHIB). *E. coli* were cultured in LB broth. Cultured bacteria were diluted to 5×10^6 cfu/ml. Diluted bacteria 50 μl was mixed with 50 μl of either BHIB (control) or the each peptide in wells of a Nunc™ 96 well plate (Thermo Scientific,

Table 2
Antimicrobial activity of RbMoro.

Bacterial strain	Gram	°C	MIC	IC_{50}
Escherichia coli (JM109)	(−)	37	1.9–3.9	7.8
Edwardsiella tarda	(−)	27	7.8–15.6	>1000
Streptococcus iniae (FP5228)	(+)	27	1.9	125–250
Vibrio alginolyticus (KCCM2928)	(−)	27	<0.9	15.6–31.2
V. campbellii (KCCM40684)	(−)	27	0.9	125–250
V. harveyi (ATCC14126)	(−)	27	<0.9	0.9–1.9
V. ordalii (KCCM41669)	(−)	27	>1000	–
V. vulnificus (KCTC2982)	(−)	27	1.9–3.9	7.8–15.6