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Two hepcidins from spotted scat (*Scatophagus argus*) possess antibacterial and antiviral functions *in vitro*

Lang Gui [a], Peipei Zhang [a], Qiya Zhang [b], Junbin Zhang [a, *]

[a] Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai 201306, China
[b] State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

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ABSTRACT

Hepcidins are small cysteine-rich antimicrobial peptides that play an important role in host immunity against pathogenic organisms. In this study, two hepcidins, SA-hepcidin1 and SA-hepcidin2, were cloned from spotted scat (*Scatophagus argus*), and the tissue distributions of SA-hepcidins were determined. In addition, mature SA-hepcidin peptides were synthesized to allow evaluation of their antimicrobial and antiviral functions *in vitro*. SA-hepcidin1 belongs to the *HAMP1* class and is widely expressed in all tested tissues from spotted scat, whereas SA-hepcidin2 belongs to the *HAMP2* class and present only in the liver. The synthetic SA-hepcidins had similar levels of antibacterial activity against Gram-positive and Gram-negative bacteria; however, the antibacterial activity of SA-hepcidin1 was stronger than that of SA-hepcidin2. The antiviral activities of the synthetic SA-hepcidins were assessed against *Siniperca chuatsi* rhabdovirus (SCRV) and largemouth bass *Micropterus salmoides* reovirus (MsReV) in *epithelioma papulosum cyprini* (EPC) and grass carp fin (GCF) cells. SA-hepcidin2 had antiviral activity, but SA-hepcidin1 did not. The results of this study suggest that SA-hepcidins are important multifunctional proteins in the spotted scat immune system that are involved in resistance to various pathogens.

1. Introduction

Fish secrete several types of antimicrobial peptides (AMPs), which are positively charged short molecules with antibacterial, antiviral, antifungal, antiparasitic, and immunomodulatory functions [1]. Hepcidin, a cysteine-rich AMP, has identified in many vertebrates. Unlike mammals, which have only a single copy of the hepcidin gene (with the exception of the mouse), many fish species have clusters of two or more copies of this gene. Hepcidin is produced mainly in the liver, but is also present in other tissues [2]. Human hepcidin is a 25-amino-acid AMP that also functions as a regulator of iron metabolism [3,4]. Secret AMPs with varying structures can be used by the pharmaceutical industry to develop novel drugs to treat drug-resistant pathogens [5]. The antimicrobial function of hepcidin has been widely investigated in fish. Exploitation of fish hepcidins could provide a source of uniquely structured antimicrobial drugs for aquaculture.

The antimicrobial activities of hepcidins from many fish species have been demonstrated, includinghepcidins from tilapia [6–12], turbot [13,14], black porgy [15], medaka [16], orange-spotted grouper [17], rare minnow [18], gilthead seabream [19], trout [20], large yellow croaker [21], zebrafish [22], channel catfish [23], Japanese flounder [24], half-smooth tongue sole [25], and sea bass [26]. Hepcidins used in antimicrobial analyses have generally been synthetic or recombinant peptides, which have consistently demonstrated antibacterial activity against Gram-positive and Gram-negative bacteria. In addition to antibacterial activity, some fish hepcidins have been reported to possess antiviral activity. The most well-studied fish hepcidin is synthetic hepcidin TH1-5 from tilapia, which has antiviral activity against infectious pancreatic necrosis virus [27], Japanese encephalitis virus [9], and nervous necrosis virus [1,7]. Recombinant Pro-Omhep1 and synthetic Omhep1 from medaka have been shown to inhibit replication of the white spot syndrome virus [16]. Two EC-hepcidins from orange-

Abbreviations: SCRV, *Siniperca chuatsi* rhabdovirus; MsReV, *Micropterus salmoides* reovirus; EPC, *epithelioma papulosum cyprini*; GCF, grass carp fin cells; AMPs, antimicrobial peptides; CPE, cytopathic effect; MIC, minimal inhibitory concentration.

* Corresponding author. Shanghai Ocean University, Halobios Faculty, Department Aquatic and Biology, 999, Hucheng Circle road, Shanghai, China.
E-mail address: jb-zhang@shou.edu.cn (J. Zhang).

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spotted grouper inhibited replication of Singapore grouper iridovirus in fish cell lines [17]. Taken together, the results of previous studies clearly demonstrate the important function of hepcidins in the fish immune system.

In fish aquaculture, fetal viral infections of pathogens such as *Siniperca chuatsi* rhabdovirus (SCRV) and largemouth bass *Micropterus salmoides* reovirus (MsReV) lead to severe disease outbreaks with significant economic consequences. SCRV is an enveloped single-stranded RNA (ssRNA) virus with a typical bullet shape [28,29]. MsReV is a non-enveloped double-stranded RNA (dsRNA) virus, infection by which leads to a typical cytopathic effect (CPE) characterized by cell–cell fusion and syncytium formation [30]. *Epithelioma papulosum cyprini* (EPC) cells and grass carp fin (GCF) cells are susceptible to infection by SCRV and MsReV [28–30]; therefore EPC and GCF cell lines are useful for studies of the antiviral functions of fish AMPs against SCRV and MsReV.

The spotted scat (*Scatophagus argus*), an economically valued fish species cultured in China, is tolerant to a wide range of environmental conditions and resistant to diseases [31,32]; therefore, it is an ideal species in which to explore the potential uses of novel AMPs developed for aquaculture. Although numerous hepcidin genes have been amplified from several species of teleosts, the functions of most fish hepcidins have not been fully determined. In this report, *S. argus* Hepcidin 1 (SA-hepcidin2) and *S. argus* Hepcidin2 (SA-hepcidin1) genes were cloned from the spotted scat. The tissue distributions of SA-hepcidins were determined, after which the antibacterial and antiviral activities of synthetic SA-hepcidins were assessed. The results of the present study contribute important knowledge regarding the functions of hepcidins in the spotted scat immune system.

2. Materials and methods

2.1. Fishes, cells, and viruses

Spotted scat having an average weight of 10 g were obtained from Zhanjiang, Guangdong province and raised in tanks in our laboratory under controlled environmental conditions (seawater at 26 ± 2 °C). Feeding was stopped two days before the experiments were performed. GCF and EPC cells were grown in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum at 25 °C and 20 °C, respectively. The preparation of virus stocks of SCRV and MsReV was performed as described previously [28,30].

2.2. Total RNA isolation and first-strand cDNA synthesis

Total RNA was extracted from each sample using TRIzol (Invitrogen, USA). RNA integrity and DNA contamination were assessed on a 1% formaldehyde gel. The concentration of RNA in the samples was measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, USA). Only samples with 260 nm/280 nm absorbance ratios (A260/A280) ranging from 1.8 to 2.0 were used. First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit With gDNA Eraser (PerfectReal Time, TaKaRa, Japan) and stored at −20 °C.

2.3. Cloning of the gene encoding SA-hepcidins

Analysis of the transcriptome of *S. argus* constructed in our laboratory using Illumina sequencing technology revealed two transcripts that had high identities with hepcidin1 and hepcidin2, and were named as SA-hepcidin1 and SA-hepcidin2, respectively. Specific primers for SA-hepcidin1 and SA-hepcidin2 cDNAs were designed using transcriptome data containing the SA-hepcidin1 and SA-hepcidin2 sequences (Table 1). The cDNA from liver was used for cloning, which was performed following a detailed procedure described in a previous study [31]. The PCR conditions were: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension step of 7 min at 72 °C. All amplified PCR products were separated by agarose gel electrophoresis, purified, cloned into the pMD 18-T vector (TaKaRa), and transformed into TOP10 Competent *Escherichia. coli* cells (Thermo Fisher Scientific, USA) for sequencing.

Table 1
Primer sequences used for ORF amplification and RT-PCR.

Primers	primer sequences (5′–3′)
HD1-F	ACAAGAGTCACCAAAAGAG
HD1-R	GGACACACAGTTGCTACAT
HD2-F	AAGATACTGTGGTGCTCTTC
HD2-R	AGGTCATTTCTTACAAGCGT
β-actin-F	CTGTGCTGTCCCTGTATG
β-actin-R	TAGTCTGTGAGGTCACGG

2.4. Sequence analysis

Putative signal peptide prediction for the identified SA-hepcidins was performed using the SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/). Predicted processing sites for mature SA-hepcidin peptides were calculated using the ProP server (http://www.cbs.dtu.dk/services/ProP/). Amino acid sequences were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/) and aligned using BioEdit 7.0.9.0. Phylogenetic trees were constructed using MEGA 5.0 software based on the multiple-sequence alignment of the identified SA-hepcidins with reported hepcidin amino acid sequences. To estimate topological stability, 1000 bootstraps were performed for the neighbor-joining trees.

2.5. Tissue distribution of SA-hepcidins

For the cloning and tissue expression analysis, fish were anaesthetized with methane sulphonate-222 (MS-222). Brain, kidney, gill, eye, liver, spleen, notochord, skin, intestine, heart, and muscle tissue were collected from 5 individuals for the purpose of RNA extraction. Semi-quantitative reverse transcription PCR (SqRT-PCR) was conducted to assess the tissue expression patterns of SA-hepcidin1 and SA-hepcidin2 in *S. argus*. The specific primers used for SqRT-PCR are listed in Table 1. The primer sequences used for β-actin cloning are also listed in Table 1. The optimized annealing temperature and PCR conditions were as follows: 58 °C, 28 cycles for β-actin; 55 °C, 28 cycles for SA-hepcidins. The PCR amplification was performed in a final volume of 25 μL containing 16.7 μL ddH_2O, 2.5 μL 1× PCR buffer, 2.5 μL Mg^{2+} (Fermentas), 1 μL dNTPs (2.5 mM), 0.3 μL Taq DNA polymerase (TaKaRa), 0.5 μL of the forward and reverse primers (10 μM), and 1 μL of template. The PCR products were analyzed by gel electrophoresis.

2.6. Synthesis of the SA-hepcidin2 and SA-hepcidin1 peptides

Linear SA-hepcidin1 (QSHLSMCRYCCNCCRNNKGCGFCCKF) and SA-hepcidin2 (NPAGCRFCCGCCPNMIGCGVCCRF) were synthesized with purity >95% by Youke Biotechnology Co. Ltd. (Shanghai, China). The molecular mass and purity of the purified peptides were verified by mass spectroscopy and high performance liquid chromatography, respectively.

2.7. Bacterial killing assay

The antimicrobial activity of synthetic SA-hepcidins was assayed