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Molecular characterization and functional analysis of the hepcidin gene from roughskin sculpin (*Trachidermus fasciatus*)

Yingying Liu [1], Xiaodi Han [1], Xuezhao Chen, Shanshan Yu, Yingmei Chai, Tongjie Zhai, Qian Zhu*

Ocean College, Shandong University (Weihai), Weihai 264209, China

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ABSTRACT

Hepcidin is a kind of cysteine-rich antimicrobial peptide that plays a vital role in host innate immune activity and iron regulation. Here, we report the molecular characterization and functional analysis of a novel hamp1 hepcidin isoforms Tf-Hep from roughskin sculpin, *Trachidermus fasciatus*. A cDNA fragment of 988 bp with an ORF of 273 bp was obtained. The coding sequence encodes for a signal peptide of 24 amino acids coupled with a prodomain of 40 amino acids and a mature peptide of 26 amino acids. Tissue distribution analysis indicated that Tf-Hep was most abundant in the liver. It could be significantly induced post lipopolysaccharide (LPS) challenge and heavy metal exposure. The mature peptide was expressed as a 6.061 kDa fusion protein in *Pichia pastoris* GS115. The active purified recombinant protein (rTf-Hep) exhibited a wide spectrum of potent antimicrobial activity in *vitro* against 4 Gram-negative bacteria *Escherichia coli, Vibrio Anguillarum, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* and 4 Gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis, Bacillus thuringiensis, and Bacillus megaterium* with minimum inhibitory concentrations (MICs) of 5–80 μg/ml (0.825–13.2 μM). It also displayed high affinity to polysaccharides on bacteria surface including LPS, lipoteichoic acid (LTA) and peptidoglycan (PGN). We further revealed that rTf-hep was capable of agglutinating 6 of the 8 bacteria. All these results suggest that rTf-hep may be both an antibacterial effector and a pattern recognition molecule in fish immune defense. The in *vivo* bacterial treatment results demonstrated that rTf-Hep could significantly improve the survival rate of fish infected with *V. anguillarum*. Taken together, these data indicate an important role for Tf-hep in the innate immunity of *Trachidermus fasciatus* and suggest its potential application in aquaculture for increasing fish resistance to disease.

1. Introduction

Due to the lack of a well-developed adaptive immune system, fish rely heavily on their innate components for an adequate response against various pathogens and environmental challenges [1]. Antimicrobial peptides (AMPs) which are the production of numerous relatively small peptides widely distributed in nature constitute the first defense line of the host innate immune system [2]. Among those, hepcidin, also termed as LEAP-1 for liver-expressed antimicrobial peptide, is a relatively recently discovered cysteine-rich AMP. It was originally isolated by two independent groups from human plasma ultrafiltrates and urine [3,4]. Since then, hepcidin has been identified in a wide range of vertebrate species. Accumulating evidences particularly from mammalian hepcidins have indicated that it is a multifunctional molecule involving in the innate immunity (as a defense AMP) and iron regulation (as a negative hormonal regulator of iron homeostasis) [4–7].

In fish, hepcidin was firstly identified in hybrid striped bass [8]. Subsequently, it was isolated and characterized from a number of fish species including mud loach [9], zebrafish [10], spinyhead croaker [11], spotted scat [12], trout [13], rare minnow [14], turbot [15,16], channel catfish [17], convict cichlid [18], European sea bass [19,20], large yellow croaker [21], Japanese flounder [22], half-smooth tongue sole [23], *etc*. Most fish hepcidins are also predominantly expressed in the liver, although more widely distribute in other tissues such as the spleen, kidney, gill, intestine, and skin [24]. Previous studies have reported that the fish hepcidins are

* Corresponding author.
E-mail address: qianzhu@sdu.edu.cn (Q. Zhu).
[1] These authors contributed equally to this work.

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modulated by different stimuli and conditions, such as LPS [9,25–28], virus [29–31], Gram-negative and Gram-positive bacteria [8,31–34], divalent heavy metals [9,35], iron overload [9], anemia [20,36], and hypoxia [19], clearly displaying important roles in the fish immune defense and immunomodulatory process. Meanwhile, these biological functions also suggest a potential value of hepcidins in aquaculture applications to develop novel drugs to treat drug-resistant pathogens.

Unlike their mammalian counterparts which have a single hepcidin gene (with the mouse being the only known exception [37]), many teleost species are thought to have acquired multiple hepcidin gene copies attributing to gene duplication and positive Darwinian selection [38], which was suggested to be lineage- or species-specific especially in fish species of Perciformes, Pleuronectiformes and Beloniformes. These fish duplications are usually grouped into two hepcidin isoforms [39]: hamp1 isoforms, which usually presents a single copy sharing a considerable degree of structural homology with their mammalian counterparts; and hamp2 isoforms, which may exhibit additional duplication and/or divergence with different characteristics. Previous studies have demonstrated that different hepcidins may possess specific subfunction and role in the immune response and iron regulation [19,20]. Moreover, hepcidins from different fish species also have presented discrepant antimicrobial spectrums. In view of these observations, the antimicrobial activities and characteristics must be explored for each newly identified hepcidin peptide in order to provide a source of uniquely structured antimicrobial drugs for aquaculture.

Roughskin sculpin, *Trachidermus fasciatus* Heckel (Scorpaeniformes: Cottidae), is a carnivorous fish with seawater-freshwater migratory habits [40]. In china, it was widely distributed along the eastern coast and the rivers flowing into this water body [41]. However, wild populations of roughskin sculpin have encountered with serious decline since 1970s mainly due to the change of living environment and the spread of bacterial infection [41]. Therefore, aquaculture has become a great requisition for population restoration, and learning how to improve the disease resistance of roughskin sculpin is essential for fishery production. Recently, a hepcidin sequence was obtained by random sequencing of the roughskin sculpin liver cDNA library. Although numerous hepcidins have been identified, the functions of most fish hepcidins have not been fully elucidated. The results we obtained in this study will provide valuable information on hepcidin function as a molecular component of the innate immunity and are useful for applications associated with disease prevention in aquaculture.

2. Materials and methods

2.1. Fish and sample preparation

Roughskin sculpin specimens ranging from 15 to 23 g in weight, approximately 9–10 months old were used in this study. They were acclimatized in a seawater tanks with continuous aeration at a temperature of 12–14 °C for more than a week prior to experimental use. Fish with signs of disease and gross abnormalities were excluded in the study.

For the bacterial LPS stimulation, two fish groups were used. The experimental group was injected intraperitoneally with *E.* LPS (Sigma, St. Louis, MO, USA) at 0.04 mg/kg and the control group with sterile phosphate-buffered saline (PBS) at 50 μl per fish. No feed was supplied during stimulatory treatments. Samples (six fish for each time point) were taken at 0, 2, 6, 12, 24, 48, and 72 h after injection. At the same time, fish of the control group were examined as well.

For metal exposure experiment, the fish were exposed to 1.32 mg/l of heavy metal (Cu: Pb: Cd: Hg = 50:10:5:1) using a stock solutions containing the corresponding heavy metal ions. Immersion after 0, 12, 24, 48, 72, and 96 h, the expressions of Tf-hep mRNA in the skin, blood, liver, and brain were analyzed.

2.2. Total RNA extraction and cDNA synthesis

Six-randomly-chosen individuals were sampled from fish belonging to each group for the purpose of RNA extraction. The fish were euthanized and tissue samples including blood, brain, gill, heart, head kidney, intestine, liver, skin, spleen, stomach, and ovary were collected. Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Then 5 μg RNA were used for synthesizing the first strand cDNA following the SMART cDNA (BD Biosciences Clontech) method with Smart F and Oligo-anchor R as primers (Table 1). The cDNA was stored at −20 °C until use.

2.3. Sequence analysis

The Tf-Hep sequence was obtained by random sequencing of the roughskin sculpin liver cDNA library. Gene translation and prediction of the deduced protein were performed with Expasy (http://www.au. expasy.org/). The presence and location of the signal peptide was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and the protein domains were revealed with the online SMART program (http://smart.emblheidelberg.de/). The calculations of molecular weight (MW) and theoretical isoelectric point (pI) were predicated by ExPASy Compute pI/Mw (http://web.expasy.org/compute_pi/). A phylogenetic tree of selected hepcidins was constructed by neighbor-joining method implemented in MEGA program based on the sequence alignment with Clustal W.

2.4. Tissue distribution of Tf-Hep mRNA and temporal expression patterns post LPS challenge and heavy metal exposure

Quantitative real-time PCR (qRT-PCR) was conducted to assess the Tf-Hep mRNA levels in different tissues of roughskin sculpin and the transcription level changes after immune stimulation. Two gene-specific primers (HepRTF, HepRTR) for Tf-Hep were designed to amplify a fragment of 193 bp. β-actin was used as internal control, and amplified with specific primers Actin F and Actin R (Table 1). According to the manufacturer's instructions in the SYBR Premix Ex Taq Kit (Takara, Dalian, China), qRT-PCR was performed with a 7300 real-time system (Applied Biosystems, USA) in a total volume of 20 μl containing 10 μl of 2 × SYBR Premix Ex Taq™, 2 μl diluted cDNA, and 4 μl each of primers (10 μM). The amplification procedure consisted of an initial denaturation step at 94 °C for 3 min, and then 40 cycles of 94 °C for 15 s, 60 °C for 60 s followed by a final dissociation stage.

All samples were repeated in triplicate for qRT-PCR analysis. The data of expression level were calculated by $2^{-\triangle\triangle CT}$ with

Table 1
Sequences of the primers used in this study.

Primers	Primer sequence (5′–3′)
HepRTF	TCTGCCATCCCATTCACCG
HepRTR	AGAAGCCGCAGCCCTTGTAG
HepF	TACTCAGAATTCCAGAGCCACCTCTCCTTGTGC
HepR	TACTCACTCGAGGAACTTGCAGCAGAAGCCGCA
HepZF	TACTCACTCGAGAAAAGACAGAGCCACCTCTCCTTG
HepZR	TACTCAGCGGCCGCGAACTTGCAGCAGAAGCCGCA
Smart F	TACGGCTGCGAGAAGACGACAGAAGGG
Oligo anchor R	GACCACGCGTATCGATGTCGACT16(A/C/G)
Actin F	TGAGACCACCTACAACAGCATC
Actin R	GAGCCTCCGATCCAGACAG