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# Genomic organization, expression and antimicrobial activity of a hepcidin from taimen (*Hucho taimen*, Pallas)

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ABSTRACT

Hepcidin, an antimicrobial peptide, plays a crucial role in innate immune system of teleost fish. As a cysteine-rich peptide, hepcidin possesses a dual function including iron regulation and innate immunity. In the present study, a full-length hepcidin cDNA (HtHep) was cloned and characterized by RT-PCR and RACE techniques from taimen (*Hucho taimen*, Pallas), which is a type of rare, precious and cold-water fish species in China. The cDNA contains an open reading frame (ORF) of 267 bp encoding 88 amino acid (aa), with 170 bp located in the 5′ untranslated region (UTR) and 151 bp in the 3′ UTR. The genomic sequences analysis showed that the *HtHep* gene consisted of three exons and two introns (with the length 94 and 251 bp, respectively). With a predicted molecular mass of 2881.4 Da and a theoretical pI of 8.53, the deduced amino acid encodes a signal peptide of 24 aa, prodomain of 39 aa and mature peptide of 25 aa. The signal peptidase (SA-VP) and the motif RX (K/R)R of propeptide convertase suggested the cleavage site of signal and mature peptide. Eight conserved cysteine residues were also identified and formed four disulfide bonds. Pair-wise alignments showed that HtHep clustered together with two fish species of Salmonidae family (*Salmo salar* and *Oncorhynchus mykiss*) in HAMP1 branch. Quantitative RT-PCR analysis indicated that the mRNA levels of *HtHep* were detected in a wide range of tissues and the highest level was detected in the liver. Its expression was also detected early during embryonic stage and could be up-regulated in the liver when challenged with pathogenic bacteria (*Yersinia ruckeri*). The recombinant HtHep (rHtHep) had antimicrobial activity against both gram-positive (*Micrococcus lysodeikticus* and *Staphylococcus aureus*) and gram-negative bacteria (*Escherichia coli*). Our results suggested that HtHep might be involved in the innate immune defense against bacterial pathogens in taimen.



## 1. Introduction

The innate immune system is crucial to fish surviving in aquatic environments with a rich microbial flora [1]. Antimicrobial peptides (AMPs) are one of the main innate immune response components of fish defense against opportunistic pathogens [2]. As a type of AMPs, hepcidin was initially discovered in humans [3,4] and has been identified from mammals, birds, reptiles, amphibians and fishes [5]. With a dual function including innate immunity and iron regulation, hepcidin is conserved between mammals and fish at both gene sequence and protein structure [1].

Hepcidin was firstly identified from hybrid striped bass (*Morone chrysops* × *Micrococcus saxatilis*) in fish [6]. The solution structure of the hepcidin was obtained and reported, showing high similarity to human hepcidin [7]. While humans [3] and mice [8] possess one and two gene copies, fishes contain up to seven hepcidin gene copies [9]. Fish hepcidins are predominantly expressed in the liver and are also detected in other tissues [6,10,11]. The hepcidin genes in fish encode a preproprotein with a signal peptide, a prodomain and a mature peptide (a cysteine-rich peptide of 20–25 amino acids) [12], and have the same disulfide-bonding pattern [7]. Its antimicrobial activity covered different bacterial and viral pathogens, and also included some certain fungi [5]. Due to its physiological relevance, hepcidin is one of the most studied AMPs in fish [13].

Taimen (*Hucho taimen*, Pallas), belonging to *Salmonidae* family, is a type of rare, precious and cold-water fish species in China [14]. It has been listed as endangered in China since 1998 [15] and was included in the China Species Red List in 2004 [16]. Among the *Salmonidae* family, taimen grow the fastest and have the largest

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body size [17]. With the development of artificial propagation, taimen has been widely cultured in China. However, little information is available about the innate immune system and the defense mechanisms against bacterial pathogens in taimen. In this study, we analyzed the genomic and cDNA sequences (including the deduced amino acid sequence) of taimen's hepcidin (named HtHep), monitoring its basal and induced (with *Yersinia ruckeri*) expression pattern. In addition, we evaluated the antimicrobial activity of the recombinant HtHep.

## 2. Materials and methods

### 2.1. Fish and bacteria

Healthy eggs and taimen (*Hucho taimen*) with 1 age old were obtained from Bohai experimental station of the Heilongjiang River Fisheries Research Institute (Harbin, China). *Yersinia ruckeri* H01, *Escherichia coli*, *Micrococcus lysodeikticus* and *Staphylococcus aureus* were provided by the Laboratory of Fish Disease Prevention and Treatment in Heilongjiang River Fisheries Research Institute.

### 2.2. Bacterial challenge and sampling

The eggs in twenty-five embryonic stages were sampled according to zhang's report [18]. Before experimental manipulation, fish were acclimatized in the laboratory at 15 °C. The kidney, gill, liver, heart, brain, intestine, blood, eye, muscle, spleen and skin were sampled for gene cloning and expression pattern analysis. The experimental fish were divided into two groups randomly: a control group and a treated group. The treated group was challenged with *Yersinia ruckeri* H01 ($1 \times 10^6$ cfu for each individual) suspended in PBS buffer by intraperitoneal injection. The control group was intraperitoneally injected with the same volume of PBS. The livers were sampled from fish at 0, 1, 2, 4, 8, 12, 24, 48, 72 h post-injection, and frozen at −80 °C for RNA preparation.

### 2.3. Full-length genomic and cDNA cloning of HtHep

Tissues were homogenized by pulverization with liquid nitrogen and total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instruction and stored at −80 °C. The total RNA was used for cDNA synthesis with SuperScript⟨SUP⟩TM⟨/SUP⟩ III reverse transcriptase (Invitrogen⟨SUP⟩TM⟨/SUP⟩). Primers (Hep-F/Hep-R) were designed to obtain the partial sequence of HtHep based on the conserved regions of hepcidin gene sequence from *Oncorhynchus mykiss* (HQ711993.1). For the full length cDNA, a RACE was performed by using the SMART-RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) with specific primers (UPM, GSP-1R and GSP-2R).

Genomic DNA was extracted to obtain the complete genomic sequence. The specific primer sets (Hep-E1F/R and Hep-E2F/R) were designed based on the consensus regions in a multiple alignment of fish hepcidins to obtain intron sequences. All of the primers used in this study were listed in Table 1. The purified DNA fragments from PCR amplification were subcloned into the pMD18-T vector (TaKaRa) for sequencing (Shanghai Shenggong Co., Ltd.).

### 2.4. Sequence analysis

The cDNA sequence of HtHep was analyzed for the identity and similarity by BLAST online. The predicted cleavage site of the signal peptide was found by Signal P 4.1program [19]. Domain search was performed with the conserved domain search program on NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [20]. The molecular weight and isoelectric point (pI) were predicted using the Expasy software (http://web.expasy.org/protparam/) [21]. The deduced amino acid sequence was converted to PDF format with the server 3D-JIGSAW (http://bmm.cancerresearchuk.org/~3djigsaw/), then 3-dimensional model of the hepcidin were determined with Swiss-PdbViewer software and RasMol software. The deduced amino acid sequence was aligned with other fish hepcidins by BioEdit software. Phylogenetic tree was constructed with MEGA 5.05 using Neighbor-Joining method based on the Poisson-corrected distances [22]. A bootstrap analysis was performed based on 1000 replications.

### 2.5. Tissue-specific and induced expression analysis of HtHep

Total RNA was extracted from egg and tissue samples using TRIzol reagent according to manufacturer instruction. Two microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, USA). Quantitative RT-PCR was carried out on the LightCycler 480 using the LightCycler 480 SYBR Green I Master (Roche, Shanghai, China). The expression level of *HtHep* was analyzed using the $2^{-\Delta\Delta CT}$ method with β-actin as an internal control [23]. Primers (qHep-F/R) for *HtHep* were designed according to the sequences obtained in this study (Table 1). The PCR parameter was 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 54 °C for 10 s and 72 °C for 30 s. Melting curve analysis was performed over a range of 65°C–95 °C in order to verify that a single PCR product was generated at the end of the assay. Each assay was performed in triplicates.

### 2.6. Prokaryotic expression and purification of recombinant HtHep (rHtHep)

The cDNA fragment encoding the predicted mature HtHep was amplified with primers (Hep-E/S) containing *Eco*R I and *Sal* I restriction site. The PCR products were digested with *Eco*R I and *Sal* I, and then ligated into the prokaryotic expression vector pET-32a (+) and transformed into competent *E. coli* Rossetta (DE3) after sequencing. The recombinant protein was expressed with IPTG (0.5 mmoL/L) induction at 37 °C, 200 rpm for 4 h and the fusion protein was purified from the inclusion bodies. Briefly, the pellets of recombinant bacteria were ultrasonicated and centrifuged at 4 °C, 12000 rpm for 15min. The supernatants were filtered by 0.45 μm membrane and then injected into HisTrapTM FF crude column. The eluted proteins were digested by enterokinase (New England BioLabs) using HiPrepTM 26/10 Desalting. Finally, the digested proteins were purified through HisTrapTM FF crude column and desalting treatment. The concentration of the recombinant proteins was determined using a spectrophotometer.

### 2.7. Antimicrobial assay

The antimicrobial activities of rHtHep were tested against *E. coli*, *M. lysodeikticus* and *S. aureus* with lysoplate assay. Briefly, logarithmic phase bacterial cultures were diluted to $10^5$ CFU/mL in LB medium containing 1% agar. Oxford cups were placed on the surface of solidified medium in the plates. rHtHep was dissolved in PBS to 0.5 mg/mL, and 50 μL protein or PBS were added to Oxford cups in the plates. The plates were incubated at 30 °C for 24 h and the bacterial growth was observed. All tests were performed in triplicates.

## 3. Results

### 3.1. Molecular characterization of HtHep

The cDNA of hepcidin of taimen (GenBank ID: KX219746) was