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Molecular cloning and antibacterial activity of hepcidin from Chinese rare minnow (*Gobiocypris rarus*)

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ABSTRACT

Background: Hepcidins, a kind of cysteine-rich antimicrobial peptides, play important roles in host immunological processes and iron regulation, which have been identified from several fish species. The rare minnow (*Gobiocypris rarus*), an endemic cyprinid fish in China, has been used extensively as model animal in laboratory. However, little is known about its hepcidin. Here, we report the cloning and characterization of a hepcidin gene from the liver of Chinese rare minnow.
Results: The full-length cDNA of rare minnow hepcidin is 662 bp, which contains an ORF of 273 bp encoding a prepropeptide of 90 amino acid residues. The predicted prepropeptide contains three domains: a signal peptide of 24 amino acids, a prodomain of 41 amino acids, and a mature peptide of 25 amino acids. Sequence alignment showed eight conserved cysteine residues in the mature peptide, which formed four disulfide bonds in spatial structure. The deduced structure of mature peptide showed a high degree of homology to the human hepcidin. Phylogenetic analysis showed that it had a close relationship with zebrafish hepcidin, and clustered in a clade with these from Cyprinidae. Synthetic peptide of rare minnow hepcidin could inhibit the growth of Gram positive bacterium *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli* and *Aeromonas hydrophila*.
Conclusion: These results suggested that rare minnow hepcidin had typical structure of hepcidins and antibacterial activity. It could participate in innate immune response as an antibacterial agent and be used as antibiotic substance.

1. Introduction

Antimicrobial peptides (AMPs) which are widely distributed in nature constitute important components of the host innate immune system [1]. AMPs play important roles in protecting host against various microbes including bacteria, fungi, protozoa, and viruses [2]. Hepcidin, a specific AMP, has been identified from mammals, amphibians, reptiles, birds, and fishes [3,4]. It plays an important role in iron homeostasis and innate immunity [5]. Mature hepcidins containing 20–25 amino acids compose of highly disulfide-bonded (cysteine-rich) β-sheets in secondary structure. Thus, conserved cysteines in hepcidin have functional roles in its spatial conformation [6].

In teleost, hepcidins have been identified from more than 20 species, such as hybrid striped bass [7], winter flounder, Atlantic salmon [6], Japanese flounder [8], red sea bream [9], tilapia [10], turbot [11], black porgy [12,13], large yellow croaker [14], medaka [15], orange-spotted grouper [16,17], mud loach [18], miiuy croaker [19], half-smooth tongue sole [20], blunt snout bream [21], common carp [22], blotched snakehead [23], convict cichlid [24], and so on. It was mainly synthesized in liver, although smaller amounts have been detected in other tissues such as the spleen, intestine, head kidney, and skin [9]. Purified hepcidin, synthetic hepcidin peptide, and recombinant hepcidin all have antibacterial activity against Gram-positive (G+) and Gram-negative (G-) bacteria [13,14,22]. It has been reported that medaka hepcidin can inhibit the growth of the G+ bacteria *Corynebacterium glutamicum*, *Staphylococcus aureus* and the G- bacteria *Escherichia coli*, *Aeromonas hydrophila*, and *Pseudomonas stutzeri* [25]. Synthetic hepcidin peptides of orange spotted grouper can delay the growth of G- bacterium *Vibrio vulnificus* and the G+ bacterium *S. aureus* [16]. Purified hepcidin peptide of large yellow croaker exhibited strong antibacterial activity against marine vibrios [26]. In addition, previous reports have shown that synthetic hepcidin peptides can inhibit virus replication [16,27]. All these indicated the important roles of hepcidin in fish innate immune system.

The rare minnow (*Gobiocypris rarus*), an endemic cyprinid fish in China, has been used extensively as model animal in research about toxicology, fish pathology, developmental biology,

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Table 1
GenBank accession numbers of hepcidins from different species used in this study.

Gene	Species	Accession number
Hepcidin 1 isoform 1	*D. rerio*	NP_991146.1
Hepcidin 1 isoform 2		NP_001276723.1
Hepcidin isoform 1	*Cyprinus carpio*	AFR23077.1
Hepcidin isoform 2		AFY23859.1
Hepcidin isoform 3		AGO64769.1
Hepcidin	*Misgurnus mizolepis*	AEM60424.1
Hepcidin	*Megalobrama amblycephala*	AFO84706.1
Hepcidin	*Schizothorax richardsonii*	AHB79194.1
Hepcidin isoform 1	*H. molitrix*	AGZ15358.1
Hepcidin isoform 2		AGT19511.1
Hepcidin	*Systomus sarana*	CAZ68137.1
Hepcidin	*Scophthalmus maximus*	AAX92670.1
Hepcidin 1	*Psetta maxima*	CAJ34592.1
Hepcidin isoform 1	*Hypophthalmichthys nobilis*	AHB64461.1
Hepcidin isoform 2		AHB64462.1
Hepcidin	*C. idella*	AEZ51835.1
Hepcidin 1	*Maylandia zebra*	XP_004551096.1
Hepcidin isoform 1	*Oreochromis niloticus*	XP_003450578.1
Hepcidin isoform 2		XP_003453536.1
Hepcidin 1	*Neolamprologus brichardi*	XP_006791782.1
Hepcidin	*Alphestes immaculatus*	AER00227.1
Hepcidin-1	*Micropterus salmoides*	ACD13023.1
Hepcidin-1	*Plecoglossus altivelis*	CBL59464.1
Hepcidin	*Larimichthys crocea*	ABC18307.1
Hepcidin-1	*Micropterus dolomieu*	ACD13025.1
Hepcidin-1 isoform 1	*Salmo salar*	NP_001134321.1
Hepcidin-1 isoform 2		ACI69335.1
Hepcidin	*Eleginops maclovinus*	ABY84822.1
Hepcidin-1	*Pagrus auriga*	BAH03285.1
Hepcidin isoform 2	*Epinephelus moara*	ADY16662.1
Hepcidin isoform 3		ADY16663.1
Hepcidin isoform 5		ADY16665.1
Hepcidin	*Pogonophryne scotti*	ABY84821.1
Hepcidin isoform 1	*Oncorhynchus mykiss*	CDQ60733.1
Hepcidin isoform 2		CDQ60734.1
Hepcidin isoform 3		ADU85830.1
Hepcidin isoform 1	*Notothenia angustata*	ABY84825.1
Hepcidin isoform 2		ABY84832.1
Hepcidin	*Takifugu rubripes*	XP_003965681.1
Hepcidin	*Morone chrysops*	AAM28440.1
Hepcidin	*Tor putitora*	AGM90578.1
Hepcidin isoform 1	*Paralichthys olivaceus*	BAE06233.1
Hepcidin isoform 2		BAE06235.1
Hepcidin isoform 3		AAT01563.1
Hepcidin	*C. semilaevis*	AFK93414.1
Hepcidin isoform 1	*I. punctatus*	NP_001188323.1
Hepcidin isoform 2		NP_001187130.1
Hepcidin isoform 1	*Oryzias melastigma*	AEG78327.1
Hepcidin isoform 2		ADM83600.1
Hepcidin	*Solea senegalensis*	BAG69595.1
Hepcidin isoform 1	*Miichthys miiuy*	AEK98541.1
Hepcidin isoform 2		AEK98542.1
Hepcidin isoform 3	*Lates calcarifer*	ADU87111.1
Hepcidin isoform 2		AEO51037.1
Hepcidin isoform 1		AEO51036.1
Hepcidin	*Poecilia formosa*	XP_007553878.1
Hepcidin	*Channa maculata*	AFN73128.1
Hepcidin	*Xiphophorus maculatus*	XP_005808614.1
Hepcidin 1	*Xenopus* (*Silurana*) *tropicalis*	NP_001090729.1
Hepcidin	*Chlorophthalmus bicornis*	AFQ32274.1
Hepcidin	*Crocodylus siamensis*	ADA68357.1
Hepcidin	*Amatitlania nigrofasciata*	AHF46363.1
Hepcidin isoform 1	*Gadus morhua*	ACA42769.1
Hepcidin isoform 2		ACA42770.1
Hepcidin isoform 1	*Lycodichthys dearborni*	ABY84842.1
Hepcidin isoform 2		ABY84843.1
Hepcidin	*Tachysurus fulvidraco*	ABX46065.1
Hepcidin isoform 1	*Monopterus albus*	ADK79123.1
Hepcidin isoform 2		ACU26539.1
Hepcidin-1	*Lepisosteus oculatus*	XP_006641712.1
Hepcidin	*Oryzias latipes*	XP_004078365.1
Hepcidin 2	*Astyanax mexicanus*	XP_007239985.1
Hepcidin	*Ictalurus furcatus*	AAX39714.1
Hepcidin	*Zanclus cornutus*	AFQ32275.1
Hepcidin	*Tarsius syrichta*	XP_008050131.1
Hepcidin-1	*Latimeria chalumnae*	XP_005995729.1
Hepcidin	*Homo sapiens*	P81172.2
Hepcidin	*Mus musculus*	NP_115930.1
Hepcidin	*Rare minnow*	

and genetics [28,29]. In this study, a hepcidin gene was cloned from the rare minnow. Antibacterial activity of its synthetic peptide was determined.

2. Materials and methods

2.1. Bacterial strains and fish

Rare minnow (*G. rarus*), with an average weight of 0.58 ± 0.06 g, were obtained from Institute of Hydrobiology, Chinese Academy of Sciences. They were cultured at 25 ± 1°C with 12 h/12 h dark/light cycle. *A. hydrophila* (strain GIM 1.172; Guangdong Microbiology Culture Center) was grown in nutrient agar at 30°C. *S. aureus* (ATCC6538) and *E. coli* (strain GIM1.571; Guangdong Microbiology Culture Center) were grown in LB medium at 37°C.

2.2. RNA extraction and cDNA cloning

For RNA extraction, liver tissue was collected from three individuals to provide enough RNA. Total RNA was extracted with Trizol reagent (Invitrogen) following the manufacturer's protocol.

For RT-PCR, primers (F: 5′-ACAGCAGRHDSARGATGAGCATCA-3′/R: 5′-TTTRCAGCART ATCCRCAGCCTTT-3′) were designed based on homologue sequences of other fish hepcidin genes. cDNA synthesis was performed using M-MLV Reverse Transcriptase (Promega) as described previously [30]. PCR products were purified and ligated into pMD18-T vector (TaKaRa). Three clones were sequenced to obtain the cDNA sequence.

5′-RACE and 3′-RACE with gene specific primers (5′GSP: 5′-AACT CTGGAGGTTGGTCTTCTCCCG-3′/3′GSP: 5′-GTTTAAAACGGGTATAAA TGCAGGC-3′) were performed to obtain the full length sequence of rare minnow hepcidin. SMART RACE cDNA Amplification Kit (Clontech, USA) was used in this step. The PCR used the following conditions: 5 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min; followed by 25 cycles of 94°C for 30 s, 68°C for 30 s, 72°C for 3 min. PCR products were ligated into pMD18-T vector (TaKaRa, Japan) and sequenced.

2.3. Sequence analysis and model structure building

Amino acid sequences of hepcidin from other species were retrieved from the GenBank (NCBI). The accession numbers were collected in Table 1. Nucleotide sequences and deduced amino acid sequences were analyzed using the EditSeq program (DNASTAR, USA). Multiple sequence alignments were conducted using the Clustal × 1.83 program. Sequence identities were calculated using the Clusta W method in the MegAlign program. Neighbor-joining phylogenetic trees were constructed using the Poisson correction models with 1000 bootstrap replicates in MEGA 6.0 [31].

The structural model of rare minnow hepcidin was constructed by SWISS-MODEL [32]. The NMR structure of human hepcidin (PDB accession number: 2KEF) was used as template [33]. The molecular graphics system PyMOL was used to render figure.