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Comparative analysis of two ferritin subunits from blunt snout bream (*Megalobrama amblycephala*): Characterization, expression, iron depriving and bacteriostatic activity

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ABSTRACT

Iron is an essential microelement for almost all living organisms, while an excess of iron is toxic, thus maintenance of iron homeostasis is vital. As iron storage protein, ferritin plays an important role in iron metabolism. In the present study, we cloned and characterized the ferritin H subunit from *Megalobrama amblycephala*, termed as *MamFerH*. An iron-responsive element (IRE) was predicted in the 5′ untranslated region (UTR) of *MamFerH*, while its bulge structural was different from that of the reported ferritin M subunit (*MamFerM*). The *MamFerH* and *MamFerM* genes exhibited similar expression patterns during early development with specifically high expression post hatching, whereas their tissue expression patterns were different. Specifically, *MamFerM* was highly expressed in the spleen, liver and kidney, while *MamFerH* was predominantly expressed in the blood and brain, indicating their different functions. In addition, the expression of the two genes was induced upon *Aeromonas hydrophila* infection at both transcriptional and translational levels, and MamFerH was more efficient. Immunohistochemistry and immunofluorescence analysis confirmed their significant changes at protein level and distribution in the liver post infection, indicating their participation in host immune response. Furthermore, bacteriostatic experiment revealed that recombinant MamFerH displayed more significant inhibitory effect on the growth of *A. hydrophila*.

1. Introduction

Iron is an essential microelement for almost all living organisms for its vital roles played in several biological processes, such as electron transport, DNA and neurotransmitter synthesis [1–3]. However, an excess of ferrous ions is toxic, as it can promote the formation of reactive oxygen species (ROS), which will provoke oxidative cell damage [1,4]. Thus, maintenance of iron homeostasis is essential and ferritin is one of the most important proteins in this process.

Ferritin is usually composed of 24 subunits that form a hollow protein shell, and it is capable of mineralizing up to 4500 iron atoms in the cavity [1,2]. In mammals, the 24-mer protein shell is made up of two functionally and genetically distinct subunits: heavy (H) and light (L) subunit. The H subunit contains 7 conserved residues that have been considered to be associated with ferroxidase activity, which is important for Fe^{2+} oxidation, whereas L subunit functions in iron nucleation and protein stability [5]. The processes of iron storage involve Fe^{2+} oxidation in the ferroxidase center, Fe^{3+} migration and binding to the nucleation sites for mineralization [1]. In consistent with their distinct functions, H and L subunits exhibit highly differential patterns of tissue distribution.

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H-rich ferritins are characteristic of organs with high iron turnover, such as the heart and brain (with subunit ration, 20H:4L). L-rich ferritins are characteristic of organs with high iron storage, such as the spleen and liver (with subunit ration, 2–3H:22-21L) [1,2,6]. Recently, a third ferritin (middle subunit) has been identified in lower vertebrates, such as fish and amphibians [4,7], which is considered to possess the characteristic features of both ferritin H and L.

Ferritins are mainly located in the cytoplasm, and low concentrations could be detected in blood plasma [1,8]. Recently, several studies have reported the mitochondria [9,10] and nuclear localization of ferritins [2]. Ferritin expression can be regulated at both transcriptional and translational levels [1,2]. At the transcriptional level, ferritin expression can be affected by a range of factors, including oxidative stress [11], temperature stress [12], heavy metals [3], cytokines [13,14] and pathogen infection [12]. The expression of ferritin can be regulated by intracellular iron levels at the translational level [1,15]. This regulation is mediated by the specific mRNA-protein interactions between iron-responsive elements (IREs) and iron-regulatory proteins (IRPs). IRPs serve as cellular iron sensor, gaining high affinity for iron-responsive elements (IREs) located in the 5'-untranslated regions (UTR) of ferritin mRNA in iron-deprived cells. The IRP/IRE complex blocks the binding of ribosome to ferritin mRNA and then inhibits translation of the ferritin mRNA [15].

Ferritins have been identified in various species from prokaryote to eukaryote [1,16]. To date, two types of ferritin subunit, H and M, have been identified in several fish species, which may assemble into heteropolymers in various proportions [17]. However, very limited studies have been conducted to compare the molecular characterization and functions of ferritins from the same species [18]. *Megalobrama amblycephala*, belonging to *Megalobrama*, Cyprinidae, one of the major freshwater aquaculture species in China, is under threat of the bacterial septicemia caused by *Aeromonas hydrophila* infection [19]. To better understand the immune roles of ferritin subunits in the *M. amblycephala* immune defense system, we cloned and characterized the ferritin H subunit from *M. amblycephala* in the present study. In addition, we examined the expression patterns of ferritin H and M subunits during embryogenesis, in different tissues, and after infection with *A. hydrophila*. Finally, we compared the iron depriving and bacteriostatic activity of the two recombinant *M. amblycephala* ferritins.

2. Materials and methods

2.1. Samples collection

All the experimental procedures involving fish were approved by Institutional Animal Care and Use Committee of Huazhong Agricultural University (HZAU). The experimental fish were anesthetized with MS 222 at 100 mg/L before dissection. All samples were flash-frozen in liquid nitrogen and stored at −80 °C for further use.

Healthy adult *M. amblycephala* (400 ± 30 g), obtained from Ezhou breeding base of HZAU, were temporarily cultured for two weeks prior to experimental manipulation. To determine the expression of target genes in various tissues, 9 tissues including liver, spleen, kidney, intestine, gill, brain, heart, muscle and blood from 6 *M. amblycephala* were collected, respectively.

Fertilized eggs generated by artificial fertilization from 5 parental *M. amblycephala*, were cultured in the recirculation water system under water temperature of 25 ± 1 °C. To determine the expression of target genes during early developmental stages, embryo at 0, 2, 6, 12, 19, 26, 32 hpf (hours post fertilization), and larval fish at 2, 6 and 15 dph (days post hatching) were collected.

Bacterial challenges were performed as described previously [20]. Briefly, 600 juvenile fish (14.6 ± 0.6 g) were randomly arranged to challenge and control groups, then injected intraperitoneally with 0.1 mL *A. hydrophila* (1×10^7 CFU/mL, half lethal dosage) or 0.6% normal saline, respectively. 30 individuals (divided into 3 pools) from each group were randomly dissected to remove the liver, spleen and kidney at 0, 4, 24, 72 and 120 h post injection.

Grass carp liver cell line (L8824) was cultured in Medium 199 containing 10% fetal bovine serum (Gibco, CA, USA) and penicillin and streptomycin (100 U/mL). L8824 cells were incubated with 40 μM $FeSO_4$ (Sigma, St. Louis, MO, USA) for 0, 3, 6, 9 and 12 h, respectively. After incubation, the cells (6-well plate each) were collected for RNA extraction.

2.2. RNA extraction and cDNA synthesis

Samples were homogenized under liquid nitrogen and total RNA was extracted using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's instructions. The quality and concentration of total RNA were measured by using agarose gel and NanoDrop 2000 (Thermo Scientific, Delaware, USA), respectively. The first strand cDNA was synthesized from 1 μg total RNA by using the PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa) following the manufacturer's protocol and stored at −20 °C for further use.

2.3. Molecular cloning of target genes

Partial cDNA fragments of *M. amblycephala* ferritin H subunit (*MamFerH*) was obtained from *M. amblycephala* expression sequence tags (EST) sequences [21], and verified via PCR amplification and DNA sequencing. To obtain full length cDNA sequences, 3′ and 5′ rapid-amplification of cDNA ends Polymerase Chain Reaction (RACE-PCR) were performed using SMART™ RACE cDNA Amplification Kit (TaKaRa), and the primers were listed in Supplemental Table 1. The 3′-RACE and 5′-RACE PCR products were cloned into pGEM®-T Easy Vector (Promega, Fitchburg, WI, USA) and sequenced in Beijing Genomics Institute (Shenzhen, China). The full length of cDNA sequence was assembled using Seqman software.

2.4. Sequence and phylogenetic analysis

The deduced amino acid of the MamFerH was predicted by Open Reading Frame Finder on NCBI website (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Theoretical molecular weight and isoelectric point were calculated using the online software ProtParam (http://web.expasy.org/protparam/). The iron-responsive elements (IREs) were predicted using Web Servers for RNA Secondary Structure Prediction (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html). The conserved domains were predicted using the ExPASy Prosite database (http://prosite.expasy.org). Multiple sequence alignment was conducted using CLUSTAL W 2.1.

In order to analyze the evolutionary context of *M. amblycephala* ferritins (MamFers), nucleotide sequences of various vertebrate ferritins (Accession No. were listed in Supplemental Table 2) retrieved from GenBank were used for phylogenetic analysis. The phylogenetic tree was constructed by MEGA 5.10 program using the maximum likelihood method [22].

2.5. Quantitative real-time PCR analysis

Expression patterns of *MamFers* were analyzed using quantitative real-time PCR (qRT-PCR) as described in previous study [20].