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Molecular properties and immune defense of two ferritin subunits from freshwater pearl mussel, *Hyriopsis schlegelii*

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ABSTRACT

Ferritin is a conserved iron-binding protein involved in cellular iron metabolism and host defense. In the present study, two distinct cDNAs for ferritins in the freshwater pearl mussel *Hyriopsis schlegelii* were identified (designated as HsFer-1 and HsFer-2) by SMART RACE approach and expressed sequence tag (EST) analysis. The full-length cDNAs of HsFer-1 and HsFer-2 were of 760 and 877 bp, respectively. Both of the two cDNAs contained an open reading frame (ORF) of 522 bp encoding for 174 amino acid residues. Sequence characterization and homology alignment indicated that HsFer-1 and HsFer-2 had higher similarity to H-type subunit of vertebrate ferritins than L-type subunit. Analysis of the HsFer-1 and HsFer-2 untranslated regions (UTR) showed that both of them had an iron response element (IRE) in the 5′-UTR, which was considered to be the binding site for iron regulatory protein (IRP). Quantitative real-time PCR (qPCR) assays were employed to examine the mRNA expression profiles. Under normal physiological conditions, the expression level of both HsFer-1 and HsFer-2 mRNA were the highest in hepatopancreas, moderate in gonad, axe foot, intestine, kidney, heart, gill, adductor muscle and mantle, the lowest in hemocytes. After stimulation with bacteria *Aeromonas hydrophila*, HsFer-1 mRNA experienced a different degree of increase in the tissues of hepatopancreas, gonad and hemocytes, the peak level was 2.47-fold, 9.59-fold and 1.37-fold, respectively. Comparatively, HsFer-2 showed up-regulation in gonad but down-regulation in hepatopancreas and hemocytes. Varying expression patterns indicate that two types of ferritins in *H. schlegelii* might play different roles in response to bacterial challenge. Further bacteriostatic analysis showed that both the purified recombinant ferritins inhibited the growth of *A. hydrophila* to a certain degree. Collectively, our results suggest that HsFer-1 and HsFer-2 are likely to be functional proteins involved in immune defense against bacterial infection.

1. Introduction

Ferritin is physiologically important for organisms in the regulation of iron homeostasis, which attributes to its capacity to bind iron [1]. Since it was first described by Laufberger in 1937 [2], ferritin has been extensively investigated with respect to its biological functions as well as structural characteristics. Recently, with increasing interests focusing on discovery of unknown properties of invertebrate ferritin, ferritin has been characterized from a various of seawater mollusks including *Crassostrea gigas* [3], *Haliotis discus discus* [4], *Meretrix meretrix* [5], *Haliotis rufescens* [6], *Saccostrea cucullata* [7] and *Argopecten irradians* [8], exhibiting its roles in antioxidative capacity and immune defense against bacterial infection.

A common architecture of ferritin has been conserved in a wide range of species including bacteria, plants and animals, consisting of a spherical molecule composed of 24 subunits with high iron-binding capacity (4500 iron atoms) [9]. Higher vertebrates usually have two different subunits known as H-chain and L-chain ferritin, which exist in spherical protein shell in different ratios depending on the tissue type and physiological status of the cell [10]. The H-chain ferritin catalyzes the oxidation of Fe (II) by its ferroxidase activity, while the L-chain ferritin lacks ferroxidase activity but possesses several negatively charged residues on the cavity surface of the ferritin shell that facilitate ferrihydrite nucleation [3,8,29]. Both H-chain and L-chain ferritin genes possess an iron response element (IRE) sequence in their 5′-untranslated region, which functions in ferritin regulation [11]. In addition to H and L, a third

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type of ferritin, which was named the *middle subunit* (M), has been identified in lower invertebrates (mainly in fish and amphibians) [8,28]. Unlike H and L subunits, M subunit possesses both the ferroxidase center of H subunit and the iron nucleation site of L subunit characterized in mammals [29].

One role of the ferritin in protection against oxidative damage was indicated by the finding that it inhibited the formation of reactive oxygen species (ROS) [12,13]. Ferritin was also considered as a regulatory factor involved in the regulation of cell proliferation [14], apoptasis [15], protein translation [16,17] and apolipoprotein B secretion [18]. The pearl oyster (*Pinctada fucata*) ferritin was reported to be involved in shell formation by iron storage [19]. Gradually, more evidence emerged for a role of ferritin during infection and inflammation as increased ferritin expression was observed under these circumstances [8,20–22], implying the property in immune response. The tactic of how ferritin participates in host innate immune defense was referred to as iron-withholding strategy [23], which suggests that the ferritin may compete with bacteria for host iron and thus inhibit their growth. Taking into account the role of ferritin in innate immunity, it is reasonable that ferritin has been attracting increasing attention for its effective antimicrobial mechanism, and has been brought into the forefront of innate immunity research.

Either in vertebrates or invertebrates, ferritin has been associated with innate immune response since the synthesis of ferritin can be regulated at both transcriptional and translational level [13,24,25]. Just as the ferritin structure is highly conserved in bacteria, plants and animals, so are the basic stimuli that can regulate its expression: heavy metal (Fe, Cu, Cd) [7,26,27], bacterial pathogens and poly(I:C) [27–29], and thermal stress [6,30], which suggested that ferritin has a broad antimicrobial spectrum involving in immune defense against infections of both bacterial and viral pathogens. Understanding for translational regulation of ferritin focuses on interaction between IRE and iron regulation proteins (IRP1 and IRP2), since the formation of the IRE/IRP complex blocks ribosome binding and translation [2]. In vitro studies showed that the purified recombinant ferritin exhibits apparent bacteriostatic effect [29]. Compared to numerous research achievements in seawater mollusks suggesting the roles of ferritin in antioxidative capacity and immune defense, little information is available on the molecular characteristics and mode of action of ferritin during pathogen infection for freshwater mollusks, especially the commercially cultured freshwater pearl mussels.

Hyriopsis schlegelii, which was introduced into China from Lake Biwa of Japan in 1998, has been extensively applied in pearl-producing industry for its high disease resistance and excellent quality of produced pearl. In the present study, we have identified two ferritin subunits from *H. schlegelii*, furthermore, we examined the tissue distribution profile of both ferritins and their transcriptional regulation after stimulation with bacteria *Aeromonas hydrophila*. And further bacteriostatic assay demonstrated that both of the two recombinant ferritins possess some antibacterial effect. To our knowledge, it was the first time to report that two distinct ferritin subunits were characterized involved in immune response in freshwater pearl mussels.

2. Materials and methods

2.1. Samples and challenge experiment

Healthy *H. schlegelii* individuals averaging 150.0 ± 10.4 mm in shell length were collected from Fuzhou Hongmen Reservoir Exploitation Corporation, Jiangxi Province, China, and were acclimatized at 23 ± 2 °C in aerated freshwater for one week before processing. The bacteria *A. hydrophila* was presented by the State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences. For challenge experiment, *A. hydrophila* was cultured in Luria-Bertani broth (LB) medium at 37 Cto mid-logarithmic phase and resuspended in phosphate-buffered saline (PBS). Total 70 mussels were randomly divided into challenge and control groups (35 individuals each group). In the challenge group, 50 μl of live *A. hydrophila* (3×10^9 CFU ml^{-1}) was injected into the adductor muscle of *H. schlegelii*, and 5 individuals were randomly sampled at 0, 2, 4, 8, 16, 24 and 48 h post-infection, respectively. The control group was injected with 50 μl PBS instead. Haemolymph from the challenge and control groups was collected from the adductor muscle by syringe and was immediately centrifuged at 4000 g for 15 min at 4 °C to harvest the hemocytes. Hepatopancreas and gonad tissues were also collected for bacteria induced expression analysis, and tissues from five individuals at each sampling point were ground in a liquid nitrogen bath and pooled together at equal amount. Meanwhile, five healthy *H. schlegelii* individuals without infection were dissected to obtain hepatopancreas, gonad, adductor muscle, axe foot, mantle, intestine, kidney, heart, gill and hemocytes for tissue expression profile analysis. All the collected samples were dissolved in 1 ml Trizol Reagent (Invitrogen) and stored at −80 °C for subsequent use.

2.2. Total RNA extraction and cDNA synthesis

Total RNA extraction was performed using Trizol Reagent (Invitrogen) according to the manufacturer's protocol, and then it was dissolved in 30 μl RNase-Free Water (TaKaRa). The quantity and purity of the extracted RNA were measured at 260 and 280 nm using a Micro-spectrophotometer (Thermo), and RNA integrity was checked by 1% agarose gel electrophoresis. Prior to cDNA synthesis, DNase treatment with RQ1 RNase-free DNase (Promega) was carried out to eliminate the genomic DNA contamination following the manufacturer's instruction. For quantitative real-time PCR reactions, 5 μg of DNase-treated RNA from different tissues were submitted to cDNA synthesis using M-MLV Reverse Transcriptase (Promega) and oligo$(dT)_{18}$ primer in a 25 μl reaction volume. The generated cDNA samples were diluted 1:10 in nuclease-free water when used as a template.

2.3. Cloning of full-length ferritin cDNA

A cDNA library was constructed from the hemocytes of *H. schlegelii* challenged with *A. hydrophila*, using SMART™ cDNA Library Construction Kit (Clontech) [31]. Random screening of the library by colony PCR using M13 forward and reverse primers was performed, and two ESTs with complete 3′-end homologous to ferritin were found by BLAST on NCBI, designated as HsFer-1 and HsFer-2.

The 5′-ends were obtained using rapid amplification of cDNA ends (RACE) approach with the SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's recommendation. The first-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Invitrogen) and primers SMART Oligo and 5′-CDS (Table 1) with 3 μg of total RNA from hemocytes as template. Two pairs of gene specific primers (GSP1 and NGSP1 for HsFer-1, GSP2 and NGSP2 for HsFer-2) (Table 1) were designed based on the known EST sequences above. For 5′-end of HsFer-1, the first-run PCR was performed using primers GSP1 and universal primer mixture UPM (Table 1). The second-run PCR was performed using primers NGSP1 and universal primer mixture UPM (Table 1) with the last PCR products diluted by ddH_2O as template. Two rounds of PCR were carried out with the touchdown PCR program: 5 cycles of 94 °C for 30 s, 72 °C for 3 min, 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 3 min, and 25 cycles of 94 °C for 30 s, 68 °C for 30 s and