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A teleostean counterpart of ferritin M subunit from rock bream (*Oplegnathus fasciatus*): An active constituent in iron chelation and DNA protection against oxidative damage, with a modulated expression upon pathogen stress

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ABSTRACT

Ferritins are biological iron chelators that can sequestrate excess iron to maintain iron homeostasis in the body. Ferritins basically consist of 2 types of subunits, designated as H and L. However, another new subunit, ferritin "M" which possesses characteristic features of both the H and L subunits, was recently identified in lower vertebrates, mostly in fish. In this study, a ferritin M-like subunit from rock bream (*Oplegnathus fasciatus*) (RbFerM) was characterized at the molecular level, and its transcriptional profile was analyzed in healthy fish, as well as in pathogen- and mitogen-stimulated fish. Furthermore, its functional properties were evaluated using the recombinant protein. The complete coding sequence of RbFerM was 528 bp in length, encoding a 176-amino acid peptide with a calculated molecular mass of 20 kDa. *In silico* analysis of RbFerM revealed that it has features similar to both the mammalian ferritin subunits, H and L. Phylogenetic analysis depicted the higher evolutionary proximity of RbFerM with its fish counterparts. Quantitative real time polymerase chain reaction (PCR) analysis detected a ubiquitous transcriptional profile of RbFerM in selected tissues of rock bream, in which more pronounced expression was observed in blood and liver tissues. Significant transcriptional inductions of RbFerM were detected in liver tissues upon lipopolysaccharides (LPS), *Edwardsiella tarda*, *Streptococcus iniae*, and rock bream irido virus (RBIV) exposures in time-course immune-challenge experiments. The purified recombinant protein of RbFerM demonstrated detectable iron chelating activity that varied with the temperature. Moreover, the recombinant RbFerM rendered a detectable protection effect against iron (II) and H_2O_2-mediated DNA damage.

1. Introduction

Iron is known to be an essential trace element because it is involved in several crucial biological processes of living organisms. However, excess iron levels in cellular environments cause deleterious effects, as iron can catalyze the production of reactive oxygen species in cells, thereby resulting in oxidative stress [1]. Therefore, maintaining an iron balance is considered essential for an organism's survival. In this regard, ferritins play an important role in iron homeostasis in the body while sequestrating excess iron and releasing them in iron dearth [2].

Ferritin is a ubiquitous and highly conserved hollow spherical protein complex comprising 24 subunits, and it is capable of mineralizing approximately 4500 iron atoms inside its thick protein shell [3]. These subunits that assemble into ferritin are composed of 2 basic types of polypeptide chains, designated as H and L [4], which are encoded by 2 distinct genes [5,6]. The H

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subunits consist of catalytic iron-binding sites, which form the ferroxidase center [7], whereas L subunits bear negatively charged amino acid residues known as iron nucleation sites, which provide ligands for binding Fe^{3+} ions [8]. The proportion of H and L subunits in the ferritin protein complex can greatly vary depending on the tissue type and physiological status of the cell. L subunits are predominantly found in ferritins of liver and spleen tissues, whereas H subunits are mainly found in ferritins of the heart and kidney [9]. Ferritin recognizes and binds Fe^{2+} ions in the ferroxidase center, where Fe^{2+} ions are oxidized by di-oxygen to Fe^{3+} [10]. Subsequently, Fe^{3+} binds to the nucleation sites of the L subunit for mineralization [8]. However, H-rich ferritins are known to be more active in iron metabolism than L-rich ferritins [7]. Moreover, in order to protect DNA from iron toxicity, H ferritins can translocate to the nuclei in some cell types or can be actively secreted to exert a wide array of biological functions [4].

Recent studies have revealed a novel subunit of the ferritin complex known as M, which is encoded by a single distinct gene and possesses active sites characteristic of both H and L subunits. The ferritin M subunit has been identified in lower vertebrate lineages, including piscines [4,11–13]. Ferritins are abundantly found in their cytosolic form; however, they can also be encountered in mammalian mitochondria and in the nuclei of plant plastids, as well as in their secreted forms in insects [4].

Recent investigations on the function of ferritin have revealed its multiple roles in organisms apart from iron homeostasis. Ferritins have also been shown to be involved in cell activation, development, immunity, and angiogenesis in animals [14–17], while orchestrating the cellular defense mechanisms against stress and inflammation [18].

Ferritin expression is regulated at both the transcriptional and posttranscriptional levels by iron response proteins, which control the amount of the protein that is expressed in or is secreted from cells [19–21]. Ferritin transcription can be modulated by different external factors such as iron flux [22], pathogen infections [13], xenobiotic stress [19], pH stress [23], and temperature [24], as well as internal factors such as oxidative stress [13], inflammatory cytokines, oncogenes, growth factors, and second messengers [19].

Ferritins can be found in a wide array of organisms from microorganisms to higher vertebrates, as well as in plants [25]. Its basic subunits, H and L, have previously been characterized from various animals, including fish. The ferritin M subunit has been identified and characterized from several marine teleost species, including *Cynoglossus semilaevis* [26], *Pseudosciaena crocea* [27], *Sciaenops ocellatus* [28], *Scophthalmus maximus* [13], and *Salmo salar* [11].

Rock bream (*Oplegnathus fasciatus*) is an economically important delicacy that is harvested by commercial fisheries and mariculture farming, accounting for prominent fish yields in eastern and southeastern Asia. However, various environmental factors can affect its growth and survival, some of which have even resulted in the reduction of mariculture production. Pathogen infections, especially bacterial and viral attacks, play a key role in such reductions by causing oxidative stress in fish, thereby affirming the necessity of a precise disease management strategy to ensure sustainability in fish mariculture farming [29,30]. In this regard, investigations of immune and oxidative defense mechanisms in this aqua-crop have become an effective approach for developing appropriate preventive schemes.

Herein, we identified and characterized a teleostean counterpart of the ferritin M subunit (RbFerM) from rock bream (*O. fasciatus*) and analyzed its transcriptional modulation upon pathogen infection. Furthermore, the iron chelating activity of purified recombinant RbFerM was demonstrated under different protein concentrations and temperatures, further evaluating its potent DNA protection effects against oxidative damage.

2. Materials and methods

2.1. Identification and sequence profiling

The complete cDNA sequence of RbFerM was identified from our sequence database of the previously constructed rock bream cDNA library [31] using the Basic Local Alignment Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, the identified sequence was characterized using different bioinformatics tools. Prediction of protein domains was carried out using the ExPASy-prosite database (http://prosite.expasy.org) and the MotifScan scanning algorithm (http://myhits.isb-sib.ch/cgi-bin/motif_scan), whereas other properties of RbFerM were determined using the ExPASy Prot-Param tool (http://web.expasy.org/protparam). Pairwise sequence alignment and multiple sequence alignment with orthologous sequences were performed using the EMBOSS needle program (http://www.Ebi.ac.uk/Tools/emboss/align) and the ClustalW2 program (http://www.Ebi.ac.uk/Tools/clustalw2), respectively. The phylogenetic relationship of RbFerM was determined using the neighbor-joining method and the Molecular Evolutionary Genetics Analysis (MEGA) software version 4 [32]. The reliability of the resultant phylogenetic reconstruction was tested using 1000 bootstrap replications.

2.2. Overexpression and purification of the recombinant RbFerM fusion protein

Recombinant RbFerM was expressed as a fusion protein with the maltose binding protein (MBP), and purified as described previously [33], with some modifications. Briefly, the coding sequence of the RbFerM gene was amplified using the sequence-specific primers RbFer-F and RbFer-R, which contained restriction enzyme sites for *Eco*RI and *Hin*dIII, respectively (Table 1). Polymerase chain reaction (PCR) was performed in a TaKaRa thermal cycler in a total volume of 50 μL with 5 U of ExTaq polymerase (TaKaRa, Japan), 5 μL of 10× ExTaq buffer, 8 μL of 2.5 mM dNTPs, 80 ng of template, and 20 pmol of each primer. The reaction was carried out at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR product (~530 bp) was resolved in 1%

Table 1
Oligomers used in this study.

Name	Purpose	Sequence (5′ → 3′)
RbFerM-qF	BAC library screening and q-PCR of *RbFerM*	TCAACATGGAGCTGTTTGCCTCTTACT
RbFerM-qR	BAC library screening and q-PCR of *RbFerM*	ACAACTTCAGAGCTGACATCAGCTTCT
RbFerM-F	ORF amplification (*Eco*RI)	GAGAGAgaattcATGGAGTCCCAAGTGCGTCAG
RbFerM-R	ORF amplification (*Hin*dIII)	GAGAGAaagcttTTAGCT CTT GCC CCC CAGG
Rb-βF	q-PCR for rock bream β-actin gene	TCATCACCATCGGCAATGAGAGGT
Rb-βR	q-PCR for rock bream β-actin gene	TGATGCTGTTGTAGGTGGTCTCGT