Developmental and Comparative Immunology 47 (2014) 104–114

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Molecular profile and functional characterization of the ferritin H subunit from rock bream (*Oplegnathus fasciatus*), revealing its putative role in host antioxidant and immune defense

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ARTICLE INFO

Article history:
Received 3 April 2014
Revised 1 July 2014
Accepted 3 July 2014
Available online 11 July 2014

Keywords:
Ferritin H
Rock bream
Recombinant protein
Functional analysis
Pathogen-induced expressional regulation

ABSTRACT

Ferritins are iron binding proteins made out of 24 subunits, involved in iron homeostasis and metabolism in cellular environments. Here, we sought to identify and functionally characterize a one type of subunits of ferritin (ferritin H-like subunit) from rock bream (*Oplegnathus fasciatus*; RbFerH). The complete coding sequence of *RbFerH* was 531 bp in length, encoding a 177-amino acid protein with a predicted molecular mass of 20.8 kDa. The deduced protein structure possessed the domain architecture characteristic of known ferritin H subunits, including metal ligands for iron binding, a ferroxidase center, and two iron-binding region signatures. As expected, the 5′ untranslated region of the *RbFerH* cDNA sequence contained a putative iron response element region, a characteristic regulatory element involved in its translation. The *RbFerH* gene comprised 5 exons and 4 introns spanning a 4195 bp region. Overexpressed recombinant RbFerH protein demonstrated prominent Fe(II) ion depriving activity, bacteriostatic properties, and protective effects against oxidative double-stranded DNA damage. Using quantitative polymerase chain reaction (qPCR), we found that *RbFerH* was expressed ubiquitously in the majority of physiologically important tissues in rock bream. A greater abundance of the mRNA transcripts were detected in blood and liver tissues. Upon administering different microbial pathogens and pathogen-derived mitogens, *RbFerH* transcription was markedly elevated in the blood of rock bream. Taken together, our findings suggest that RbFerH acts as a potent iron sequestrator in rock bream and may actively participate in antimicrobial as well as antioxidative defense.

1. Introduction

Iron, a vital element found abundantly in living organisms has indispensable roles in an array of biological events including metabolic redox processes. However, excessive levels of reduced or free iron in cells can lead to detrimental toxic effects. Fenton-type reactions involving in the catalysis of free Fe(II) ions in cellular environments induces reactive oxygen species (ROS)-mediated oxidative stress (Crichton et al., 2002). Therefore, proper *in vivo* regulation of free iron availability is required for the survival of living cells. This is facilitated by balancing the uptake of iron through increasing the efficacy of iron transport and storage. Ferritins play a major role in iron storage by binding excessive free Fe(II) ions in cellular environments, releasing them only in times of iron shortage (Watt, 2011). Thus, ferritin is involved in iron detoxification through its iron binding property, maintaining iron homeostasis and limiting iron bioavailability to nontoxic levels.

Ferritins are ubiquitously distributed proteins in both prokaryotes and eukaryotes. Three-dimensionally, eukaryotic ferritin is arranged into a hollow spherical protein complex consisting of 24 subunits that can mineralize approximately 4500 iron atoms inside its thick protein shell (Crichton and Declercq, 2010). In the vertebrate lineage, two ferritin subunits encoded by distinct genes have been identified (Caskey et al., 1983; Worwood et al., 1985).

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http://dx.doi.org/10.1016/j.dci.2014.07.004
0145-305X/

Heavy (H) and light (L) chain ferritin subunits (Arosio et al., 2009) have molecular masses ranging between 18 and 28 kDa. Ferritin H subunits are characterized by their unique ferroxidase center involved in Fe(II) ion binding (Lawson et al., 1991). The L subunits, on the other hand, are identified by their iron nucleation sites that allow ligands to bind Fe(III) ions. The ferritin ferroxidase center recognizes and binds Fe(II) ions that become oxidized to Fe(III) ions by dioxygen (Lawson et al., 1989). Fe(III) then binds to nucleation sites to undergo the mineralization process (Santambrogio et al., 1996). The composition ratio of H and L subunits in ferritin can vary greatly depending on the tissue type and physiological status of different cells. For instance, H-rich ferritins are abundant in heart and kidney tissues, whereas L-rich ferritins are more abundant in the liver and spleen (Arosio et al., 1976). In addition to the two main ferritin subunits, a third middle (M) subunit has been identified., The M subunit shows characteristic features and functions of both H and L subunits which is commonly found in fish and amphibians (Andersen et al., 1995; Dickey et al., 1987; Zheng et al., 2010a).

Ferritins have been identified in a diverse group of living organisms including microorganisms, plants, and most vertebrate and invertebrate species, examined. The ferritins from different species share common features (Theil, 1987). Ferritins are predominantly cytosolic proteins; however, they are also present in the mitochondria in some insects (Missirlis et al., 2006) and both mitochondria and nuclei of several mammalian cell types, protecting these organelles from iron toxicity and oxidative damage. Ferritins are also found in the plastids of plant cells and secreted forms have been identified in insects (Andrews et al., 1992; Arosio et al., 2009). Further, regarding their function in iron metabolism, ferritins reportedly play a significant role in other biological processes including cell activation, development, immune defense, and angiogenesis (Alkhateeb and Connor, 2010; Coffman et al., 2008; Parthasarathy et al., 2002; Wang et al., 2010). Expression of the ferritin subunits is tightly regulated at the transcriptional and post-transcriptional levels. Iron responsive element binding proteins have a major role in their transcriptional regulation (Outten and Theil, 2009; Theil, 2007; Torti and Torti, 2002). These regulatory mechanisms cooperatively control the amount of ferritin that is expressed in or secreted from cells. Exogenous conditions such as pathogen infection (Zheng et al., 2010a), xenobiotic stress (Torti and Torti, 2002), iron load (Wu et al., 2010), temperature stress (Salinas-Clarot et al., 2011), pH stress (Zhou et al., 2008), and endogenous factors like oxidative stress (Zheng et al., 2010a), and inflammatory cytokines (Torti and Torti, 2002) also regulate the expression of ferritin subunit genes at the transcriptional level.

As previously reported, both ferritin H and M subunits were already identified from Atlantic salmon (*Salmo salar*), from which H subunit was found to express mainly in spleen, liver and heart, whereas M chain was exclusively expressed in gonads (Andersen et al., 1995). Interestingly, three isoforms of ferritin H subunits were identified form rainbow trout (*Oncorhynchus mykiss*) and detected their cold inducible gene expression (Yamashita et al., 1996). Recent reports on teleostan ferritin M and H subunits suggests that teleostan ferritins may involve in host immune responses besides its main role in iron homeostasis, further participating in host antioxidant defense (Elvitigala et al., 2013; Liu et al., 2010; Zhang et al., 2010; Zheng et al., 2010a,b). However, detailed characterizations and functional analyses of ferritin H orthologs have been performed in a limited number of teleost species including catfish (*Ictalurus punctatus*) (Liu et al., 2010), turbot (*Scophthalmus maximus*) (Zheng et al., 2010b), sea bass (*Dicentrarchus labrax*) (Neves et al., 2009), and the Atlantic salmon (*S. salar*) (Andersen et al., 1995). Therefore, our knowledge regarding teleost ferritin H orthologs is incomplete.

Mariculture farming has become an indispensable source of foods enriched in essential amino and fatty acids. Rock bream (*Oplegnathus fasciatus*), an edible maricultured fish species, is an important aqua-crop. It accounts for a considerable fraction of the total annual fish yield, making rock bream a profitable species in the global aqua-farming industry, particularly in East and South East Asia. However, considerable production loss of this maricultured species has been incurred recently, primarily because of the prevalence of bacterial and viral infections (Do et al., 2004; Mohanty and Sahoo, 2007) Thus, development of novel disease management strategies is essential to improve rock bream aqua-farming yields. Investigation into the naturally existing host immune defense mechanisms and strategies may contribute substantially towards this effort.

In this study, we identified and molecularly characterized a ferritin H-like subunit from rock bream (RbFerH), and investigated its functional role in iron(II) deprivation, antibacterial defense, and antioxidative defense by using recombinant RbFerH protein (rRbFerH). The tissue-specific expression of *RbFerH* was analyzed in several physiologically important tissues. In addition, we examined the regulation of *RbFerH* transcription in response to bacterial and viral fish pathogens along with their mitogens.

2. Materials and methods

2.1. Identification and sequence analysis of RbFerH

To identify the full-length cDNA sequence of *RbFerH*, we analyzed rock bream DNA sequence data from a cDNA library constructed previously (Whang et al., 2011a), using the Basic Local Alignment Search Tool (BLAST) algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identified *RbFerH* sequence was characterized using several *in silico* tools. The putative complete open reading frame (ORF) of *RbFerH* was identified and the corresponding amino acid sequence was deduced using the DNAsist 2.2 software. Protein domains were predicted using the ExPASy Prosite database (http://prosite.expasy.org). Physicochemical properties were determined using the ExPASy ProtParam tool (http://web.expasy.org/protparam). Pairwise and multiple sequence comparisons of RbFerH with its homologues were performed using the EMBOSS needle (http://Ebi.ac.uk/Tools/emboss/align) and ClustalW2 (http://Ebi.ac.uk/Tools/clustalw2) programs, respectively. The phylogenetic reconstruction of RbFerH and its orthologs was generated by the neighbor-joining method using Molecular Evolutionary Genetics Analysis version 4.0 (MEGA 4.0) software (Tamura et al., 2007).

The complete gene sequence of *RbFerH* was also identified in a custom constructed rock bream random-shear bacterial artificial chromosome (BAC) genomic DNA library (Lucigen®, USA). The BAC clone bearing the *RbFerH* gene was identified using a two-step polymerase chain reaction (PCR)-based genomic library screening approach using gene specific-primers (RbFerHqF and RbFerHqR; Table 1). The putative *RbFerH*-containing clone was then sequenced using the GS-FLX™ system (Macrogen, Korea) to obtain full-length genomic sequence. Using the National Center for Biotechnology Information (NCBI) Spidey online server (http://www.ncbi.nlm.nih.gov/spidey) and previously identified full-length *RbFerH* cDNA sequences, we annotated the exon–intron arrangement. Annotated sequence information for *RbFerH* was deposited in the NCBI GenBank database (accession number KJ461740).

2.2. Expression and purification of recombinant RbFerH fusion protein

rRbFerH fused to maltose binding protein (MBP) was expressed and purified as previously described, but with some modifications (Umasuthan et al., 2011). Briefly, the coding sequence of the *RbFerH* gene was amplified using the sequence-specific primers