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Molecular characterization and tissue distribution of ferritin M in kelp grouper, *Epinephelus bruneus*

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This study, reports the identification and analysis of ferritin M chain, from kelp grouper, *Epinephelus bruneus* (EbFerM); it comprises 1004 base pair (bp), including 528 bp open reading frame (ORF) which encodes 176 amino acid (aa) residues; the calculated molecular weight is 20 kDa. The 5′-untranslated region (UTR) possesses 476 bp proceeded by a putative Iron Regulatory Element (IRE). Pair wise alignments showed that EbFerM shared 94% identity with that of *Larimichthys crocea* and *Sciaenops*. It is expressed in abundance in liver, spleen, and kidney when challenged with *Vibrio anguillarum*, lipopolysaccharide (LPS), or poly I:C.

[**Key words:** *Epinephelus bruneus*; Ferritin M; Gene expression; RT-PCR; Tissue expression]

Ferritins (FTs) are the members of ferritin-like diiron-carboxylate protein superfamily; they are composed of three subunits called heavy (H), middle (M), and light (L) chains forms (1) which are different in rates of iron uptake and mineralization. Ferritins H subunit has 7 conserved residues in Miyagi oyster (2); the L subunit which has been found only in vertebrates (3) does not possess ferroxidase activity. Furthermore, it has been reported that a third subunit type named as M chain is found in Antarctic teleosts (4). Ferritin H may suppress proliferation of T cells (5) and colony formation by normal human macrophages (6). It has been reported that ferritin is indirectly linked to the innate immune response since the synthesis of ferritin is regulated by pro-inflammatory cytokines at both transcriptional and translational levels in higher vertebrates (7). The iron levels in the cell regulates the ferritin transcription, removes the interaction between iron regulatory proteins (IRP) and iron response elements (IRE) which are located in 28 bp upstreaming in 5′-untranslated region (UTR) (8,9). Despite the various studies on ferritins in various animals and there is a paucity of work in fish to understand the relationship between cellular stress and the transcriptional level of the genes involved in immune system. To our knowledge, this is the first report demonstrating the molecular information and the transcriptional levels of ferritin M to understand immune system in kelp grouper after being challenged with *Vibrio anguillarum*, endotoxin lipopolysaccharide (LPS), and double-stranded synthetic RNA poly(I:C).

We have constructed a kelp grouper cDNA sequence database by the FLX™ genome sequencing technique. Total RNA was isolated using Tri Reagent™ (Sigma, St. Louis, MO, USA) from several tissue pools including gills, liver, muscle, heart, kidney, spleen, and intestine of three healthy kelp grouper. Then, the mRNA was purified using an mRNA isolation kit (FastTrack® 79 2.0, Invitrogen, USA). The first strand cDNA synthesis and normalization were carried out with the Creator™ SMART™ cDNA library construction kit (Clontech, USA) and Trimmer-Direct cDNA normalization kit (Evorgen, Russia). Thereafter, the GS-FLX™ sequencing of kelp grouper cDNA was performed according to the manufacturer's instructions. From the kelp grouper cDNA sequence library, a single putative EST gene namely ferritin middle chain (EbFerM) was identified during homology screening using BLAST program available in the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast).

The calculated molecular mass and theoretical isoelectric point (pI) were predicated by EditSeq in the DNASTAR software package (DNASTAR Inc., Madison, WI, USA). Similarities were compared with other known ferritin M sequences available in the NCBI databases. To obtain the open reading frame (ORF) aa sequence of EbFerM using DNAssist (version 2.2). Characteristic domains or motifs were identified using PROSITE profile database (http://kr.expasy.org/prosite/) and simple modular architecture research tool (SMART) proteomic database (http://www.smart.embl-heidelberg.de/). Percentages identity, similarity, and gap were calculated using EMBOSS pair-wise alignment algorithms. Prediction of signal peptide was accomplished using the SignalP worldwide web server. The aa sequences were aligned using the ClustalW program and the phylogenetic tree was reconstructed using

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the neighbor-joining (NJ) method by the molecular evolutionary genetic analysis (MEGA 4.1) software package with bootstrapping values taken from 1000 replicates.

Kelp grouper *E. bruneus* (23.6 ± 1.5 g) obtained from local fish farm in Jeju Island, South Korea were transported and acclimatized for 2 weeks in a recirculation culture system at Jeju National University. Fish were divided into four groups of 25 each to determine the consecutive expression of EbFerM to *V. anguillarum*, LPS, and poly I:C. For bacterial challenge, the fish were injected intraperitonealy (i.p.) 100 μl phosphate buffered saline (PBS) containing *V. anguillarum* (2.8×10^7 CFU/ml); other groups were injected i.p. with 100 μl PBS suspension with poly I:C (100 μg/ml, Sigma) or LPS (1.25 μg/ml, 055: B5 from *Escherichia coli*, Sigma). A positive control group was injected i.p. with 100 μl PBS suspensions alone. Each group was maintained in triplicate. On 3, 6, 12, 24, 48, and 72 h post-infection, three kelp groupers were sacrificed with tricaine methanesulfonate (Sigma) 1:4000 in dechlorinated water for 2 min, and the liver, spleen, kidney, gills, muscle, and heart were collected under aseptic conditions to determine the transcriptional profiles of EbFerM. For each sample, equal amounts of tissue from the same organ among the three fish were combined and used to isolate total RNA. All tissue samples were fixed in RNA later Stabilization Reagent (Ambion) and snap-frozen in liquid nitrogen immediately, and stored at −80°C until used for the total RNA isolation.

The total RNA was extracted from pooled tissues of 3 untreated control fish (PBS alone), *V. anguillarum*, LPS, or poly I:C-induced individuals using Tri Reagent™ according to the manufacturer's protocol. Originally purified RNA was diluted to 1 μg/ml concentration prior to cDNA synthesis. Briefly 2.5 μg RNA was used to synthesize cDNA from each tissue using a SuperScript III first strand synthesis system for RT-PCR (Invitrogen). The RNA was incubated for 5 min at 65°C with 1 μl of 50 μM oligo$(dT)_{20}$ and 1 μl of 10 mM dNTP. After incubation, 2 μl of 10× cDNA synthesis buffer, 2 μl of dithiothreitol (DTT, 0.1 M), 4 μl of 25 mM $MgCl_2$, 1 μl of RNaseOUT™ (40 U/μl), and 1 μl of SuperScript III reverse transcriptase (200 U/μl) were added and incubated for 1 h at 50°C. The reaction was stopped by raising the temperature at 85°C for 5 min. Then 1 μl of RNAse H was added to each synthesized cDNA and incubated for 20 min at 37°C. The resulting cDNA was diluted 10-fold before being stored at −20°C for further experiments.

The RT-PCR was carried out to analyze the mRNA expression of EbFerM, in a 20 μl reaction volume containing 4 μl of cDNA from each tissue, 10 μl of 2× TaKaRa Ex Taq™ SYBR premix, 0.5 μl of each gene-specific primer (20 pmol/μl), and 5 μl of H_2O. The semi quantitative RT-PCR cycle profile was: 1 cycle of 95°C for 10 s, followed by 35 cycles of 95°C for 5 s, 58°C for 10 s and 72°C for 20 s, and finally, 1 cycle of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The semi quantitative RT-PCR cycle profile was used for the internal reference gene, β-actin. The primers used to amplified the β-actin gene were ATF1 (5′-AGACCTTCAACACTCCTGCCATGT-3′) and ATR1 (5-TGGATCTGGCTGGCAGAGATTTGA-3′) with 183 bp size; the primers used to amplified EbFerM were FerMRTF (5-GCACAAGCTGGCTTCT-GATCATGT-3′) and FerMRTR (5′-ACAGGTACTCCGCCATCTTGTTGT-3′) with 158 bp size. The β-actin and ferritin sequences were deposited in NCBI and the accession no. JF317678 and JF317679, respectively. The baseline was set automatically using the Thermal Cycler Dice™ Real Time System Software (version 2). The relative expression was determined by means of the $2^{-\Delta\Delta CT}$ 150 method (27) with β-actin mRNA as the internal control. All data represent means ± standard deviation.

Based on ESTs analysis of the kelp grouper cDNA library, a full length cDNA from a single clone (CL72Contig1) was identified as ferritin M chain by BLAST searching in the GenBank designated as EbFerM. The EbFerM cDNA is 1004 bp in length and contains an ORF with 528 bp encoding a polypeptide of 176 aa. The calculated molecular weight of EbFerM is 20 kDa with pI of 5.04 as determined by the DNASTAR software. There is also a polyadenylation signal AATAAA at 686th to 691st position. Additionally, there is an upstream in-frame stop codon (TAA) indicating that the cDNA contains the complete coding sequence. Analysis of the 5′-UTR by the method of Durand et al. (2) demonstrated the presence of a putative 30 bp IRE at the position of 12th to 41st (GTTCTTGCTTCAACAGTGTTTGAACG-GAGC) in EbFerM cDNA. In addition, there was a putative C-terminal cystine knot domain signature between 301 and 337 bp in kelp grouper according to the PROSITE program. Using the SignalP 3.0 program, the signal peptides were found in the N-terminus of ferritin, which was cleaved at aa positions between 24th and 25th and signal peptide probability is 0.685 which yield a putative secreted mature protein. It also has the 8 characteristic aa that have ferroxidase function on the ferritin M chain as reported in mammals; its residues which serve as metal binding sites in mammalian ferritins were identified in EbFerM. There are three conserved metal ligands present in EbFerM, which have been identified as metal ligands at the M-specific ferroxidase center in mammalian ferritins. The putative IRE can be folded into a typical stem-loop secondary structure (Fig. 1A and B), which perfectly matches all IRE characteristics, including its six nucleotide loop 5′-CAGUGU-3′, its proximal stem of five paired bases, followed by a bulged cysteine, and its six nucleotide bottom stem that identified in most ferritin mRNAs. These IREs are present in majority of ferritin mRNAs; their alignment and prediction of loop structure of this newly characterized IRE from the kelp grouper shows again a primary relevant consensus with the available fish and other vertebrates species IREs like *Scophthalmus maximus, Branchiostoma belcheri, Danio rerio, Lymnaea stagnalis,* and *Homo sapiens* (Fig. 1A and B). The present results showed that the gene encoding 7 residues corresponding to the ferroxidase site were highly similar to those found by Durand et al. (2) in *Crassostrea gigas*, by Zhang et al. (10) in *Pinctada fucata* and by Wang et al. (11) in *Meretrix meretrix*.

The phylogenetic tree was constructed based on the comparison of deduced aa sequences of EbFerM in different species which showed that the fish ferritin M is more closely related with vertebrate ferritins and cluster together into one group. EbFerM shared a higher identity and similarity with other fish ferritin M than with other vertebrates by unrooted phylogenetic tree construction using neighbor-joining method (Fig. 2). The deduced aa sequence of EbFerM shared 94% identity with *Larimichthys crocea* and *Sciaenops ocellatus*, followed by 92% with *Anoplopoma fimbria*, 88% with *Oncorhynchus mykiss*, *Salmo salar*, *Caligus rogercresseyi*, *Osmerus mordax*, and *Ictalurus furcatus* of ferritin M, respectively. Iron-binding proteins, such as transferrins and ferritins, have been discovered and these have demonstrated that their iron-withholding nature may be elegantly employed to inhibit bacterial growth (12). The presence of iron-binding sites is known to affect the iron storage function in *E. coli* (13). The present study has identified that EbFerM contains metal binding sites which imply that they are assigned diverse functions in iron storage and detoxification. In the present study, a putative IRE was found at 12th to 41st position with 243 bp in 5′ UTR of EbFerM cDNA. The IRE-like elements together with the IRPs are responsible for the translational control of ferritin synthesis by iron (14). This process is mediated by the intracellular pool of labile Fe as well as by the interaction between RNA binding proteins (IRP) and the IRE in the 5′ UTR of ferritin mRNA (8). The changes in IRP binding IRE result from the formation of an iron sulfur cluster in the protein core (15).

Though the EbFerM expression was found amplified in all tissues, however a higher gene expression was found in liver and spleen between 24 and 48 h post injection/challenged with *V. anguillarum*, LPS or poly I:C (Fig. 3). The expression patterns were similar to that of the horseshoe crab which secreted ferritin H (16) and the echinoderm secreted H-like ferritin (17) during bacterial infection. There was an increase of secreted ferritin mRNA transcript in the horseshoe crab after LPS challenge between 6 and 48 h (16). The constitutive expression of EbFerM in the present study suggests that they have various functions in the biological