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Identification and molecular analysis of a ferritin subunit from red drum (*Sciaenops ocellatus*)

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ABSTRACT

Ferritin is a conserved iron binding protein existing ubiquitously in prokaryotes and eukaryotes. In this study, the gene encoding a ferritin M subunit homologue (SoFer1) was cloned from red drum (*Sciaenops ocellatus*) and analyzed at expression and functional levels. The open reading frame of *SoFer1* is 531 bp and preceded by a 5′-untranslated region that contains a putative Iron Regulatory Element (IRE) preserved in many ferritins. The deduced amino acid sequence of SoFer1 possesses both the ferroxidase center of mammalian H ferritin and the iron nucleation site of mammalian L ferritin. Expression of *SoFer1* was tissue specific and responded positively to experimental challenges with Gram-positive and Gram-negative fish pathogens. Treatment of red drum liver cells with iron, copper, and oxidant significantly upregulated the expression of *SoFer1* in time-dependent manners. To further examine the potential role of SoFer1 in antioxidation, red drum liver cells transfected transiently with SoFer1 were prepared. Compared to control cells, SoFer1 transfectants exhibited reduced production of reactive oxygen species following H_2O_2 challenge. Finally, to examine the iron binding potential of SoFer1, *SoFer1* was expressed in and purified from *Escherichia coli* as a recombinant protein. Iron-chelating analysis showed that purified recombinant SoFer1 was capable of iron binding. Taken together, these results suggest that SoFer1 is likely to be a functional ferritin involved in iron sequestration, host immune defence against bacterial infection, and antioxidation.

1. Introduction

To most living organisms, iron is an essential nutrient that is involved in many fundamental biological processes. However, iron excess can be deleterious to cells, as in the presence of oxygen, iron can promote the generation of highly reactive hydroxyl radicals through Fenton reaction, thus causing damage to cellular components [1]. As a result, iron uptake and metabolism are tightly controlled in aerobic organisms. Ferritin is a protein that has evolved to maintain iron homeostasis in a wide range of life forms, including both prokaryotes and eukaryotes [2]. Structurally, ferritin is a globular protein complex composed of 24 subunits that form a shell-like structure with a large inside cavity, which serves as an iron storage site [3]. The shell of apoferritin, which is ferritin prior to iron association, can accommodate about 4500 Fe(III) ions. Iron uptake requires a specific catalytic site named ferroxidation center, which is a carboxylate-bridged diiron center situated at the subunit four-helix bundle, where Fe(II) is oxidized to Fe(III) through a series of reactions involving dioxygen or peroxides. Fe(III) then migrates to the cavity where nucleation and mineralization take place, resulting eventually in the formation of a compact iron core [4–6]. Thus, through ferritin, potentially toxic iron is rendered harmless and sequestered in a safe deposit that can be drawn for biological needs under conditions of iron scarcity. Since the ferroxidation process utilizes as oxidants both O_2 and H_2O_2 [7], ferritin plays an important role not only in iron storage but also in antioxidation.

In mammals, two types of ferritin subunits, H and L, have been identified, which are similar in higher order structures [3]. H and L subunits are transcribed independently from distinct genes and assemble into heteropolymers in various proportions [8,9]. In isoferritins composed of mixed H and L, the two subunits perform different functions. H subunit possesses the ferroxidase center and is responsible for iron oxidization, whereas L subunit lacks ferroxidase activity but facilitates iron nucleation by providing several negatively charged residues on the inner surface of the ferritin shell [10,11]. In addition to H and L, a third type of ferritin subunit, M, has been identified in fish and amphibians [12–16]. M subunit is unique in the feature that it possesses both the ferroxidase center of H

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subunit and the iron nucleation site of L subunit. It is known that in some fish species, M subunit can form a homopolymer [16].

In animals, regulation of ferritin synthesis is exerted at both transcription and post-transcription levels [17,18]. It is known that multiple factors, such as iron [19,20], copper [21], hormones [22,23], cytokines [24,25], c-MYC [26], cAMP [27], and oxidants [28], affected the transcription of ferritin. At the translation level, one of the most clearly illustrated regulations is mediated through the so-called Iron Responsive Proteins (IRPs) I and II, which repress the translation of ferritin mRNA by binding to a specific site called Iron Regulatory Element (IRE) located at the 5′-untranslated region (UTR) of many ferritins [29,30].

Red drum (*Sciaenops ocellatus*), also known as channel bass, redfish, or spottail bass, is the only species in the *Sciaenops* genus. It is ranked as an important economic fish species and has been extensively cultured in south China since 1991. In this study, we reported the cloning and analysis of a ferritin gene, *SoFer1*, from this fish species. We found that SoFer1 possesses sequence features typical of M ferritin and exhibits an expression pattern that is tissue specific and affected by stress conditions associated with bacterial infection and exposure to iron, copper, and oxidant. Our results suggest that SoFer1 is likely to play a role in iron storage, anti-oxidation, and immune defence against microbial infection.

2. Materials and methods

2.1. Fish

Red drum (*Sciaenops Ocellatus*) were purchased from a commercial fish farm in Fujian Province, China, and maintained at 24 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before being subjected to experimental manipulation. Fish were anaesthetized with tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving injection, blood collection, and tissue collection.

2.2. Bacterial challenge and tissue collection for cDNA library construction

Streptococcus iniae G26 and *Edwardsiella tarda* TX1, both are fish pathogens, were cultured in Luria-Bertani broth (LB) [31] medium at 28 °C as described previously [32,33] to mid-logarithmic phase and resuspended in phosphate-buffered saline (PBS). Red drum (860–910 g) were randomly divided into two groups (3 fish/group) named A and B. Fish of group A were each injected intraperitoneally (i.p.) with 1×10^8 CFU of G26 and TX1, while fish of group B were injected with PBS. At 24 h post-infection, the fish were sacrificed with a lethal dose of tricaine methanesulphonate, and the spleen, liver, and head kidney were collected under aseptic conditions. Tissues from each fish were pooled together at equal amount and frozen in liquid nitrogen. The pooled tissues were grounded in a mortar, and equal amounts of grounded tissues from each fish were mixed and used for RNA extraction.

2.3. RNA extraction and cDNA library construction

Total RNA was isolated from the pooled fish tissues with the RNAprep Tissue/Bacteria Kit (Tiangen, Beijing, China). The RNA was used for the construction of cDNA library with the Super SMART PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instructions.

2.4. DNA sequencing and cloning of SoFer1

Plasmid DNA was isolated from ~2000 clones of the cDNA library and subjected to DNA sequencing with the standard T7 primer. Blast analysis indicated that one of the expressed sequence tags shares high sequence identities with known ferritin subunits. The 5′ and 3′ ends of this ferritin homologue (named SoFer1 for *S. ocellatus* ferritin 1) were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer's recommendations. Briefly, for 3′ end RACE, the first-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and primer 3′-CDS {5′-AAGCAGTGGTATCAACGCAGAGTAC(T)30VN-3′; V = A, G, or C; N = A, C, G, or T}. The first-run PCR was performed with the specific primer RDF-F1 (5′-GATGGCAGAGTACCTATTTGAC-3′) and the universal primer UPM (5′-CTAATACGACTCACTATAGCAAGCAGTGGTATCAACGCAGAGT-3′ and 5′-CTAATACGACTCACTATAGGGC-3′). The second-run PCR was performed with the specific primer RDF-F2 (5′-CCGAGGATAGAGCCTGGAAT-3′) and the nested universal primer NUP (5′-AAGCAGTGGTATCAACGCAGAGT-3′). The PCR was carried out with the following program: 35 cycles at 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 50 s, followed by an extension at 72 °C for 10 min. For 5′ end RACE, the first-strand cDNA was synthesized using reverse transcriptase (as above), Oligo(dG)-adaptor primer (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′), and Oligo(dT)-adaptor primer {5′-(T)25VN-3′; N = A, C, G, or T; V = A, G, or C}. The first-run PCR was performed with the specific primer RDF-R1 (5′-GCAGCTTCTGGGCATGCT-3′) and UPM. The second-run PCR was performed with the specific primer RDF-R2 (5′-TCACGGGAGAAGTAAAAGGC-3′) and NUP. The PCR condition was the same as that described above. The PCR products were cloned into the pMD18-T vector (Takara, Dalian, China) and subjected to sequence analysis.

2.5. Sequence analysis

The cDNA and amino acid sequences of SoFer1 were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System. Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The calculated molecular mass and theoretical isoelectric point were predicated by EditSeq in the DNASTAR software package. Multiple sequence alignment was created with the Clustal program. Phylogenetic analysis was performed with Clustal and the Neighbor-joining (NJ) algorithm of MEGA 4.0.

2.6. Quantitative real time reverse transcriptase-PCR (qRT-PCR) analysis of SoFer1 expression in fish tissues

Brain, heart, gill, kidney, spleen, liver, muscle, and blood were taken aseptically from five fish and used for total RNA extraction as described above. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in an ABI 7300 Real-time Detection System (Applied Biosystems, Foster City, CA, USA) by using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [34]. Each assay was performed in triplicate with β-actin mRNA as the control. The primers used to amplify the β-actin gene were ATF1 (5′-GCCCCACCTGAGCGTAAAT-3′) and ATR1 (5′-CGGACTCATCATACTCCTGCTT-3′); the primers used to amplify *SoFer1* were RfrF1 (5′-CGTCAAGAAACCAGAGCGTG-3′) and RfrR1 (5′-ATGCGGGAGAGGTTGGAAAT-3′). All data are given in terms of

relative mRNA, expressed as means plus or minus standard errors of the means (SE).

2.7. Expression of SoFer1 in response to bacterial challenge

S. iniae G26 and *E. tarda* TX1 were cultured in LB medium and resuspended in PBS as described above to 2×10^7 CFU ml^{-1} and 5×10^6 CFU ml^{-1}, respectively. Red drum (~10 g) were divided randomly into three groups (5 fish/group) and injected i.p. with 100 μl of G26, TX1, or PBS. Fish were sacrificed at various times post-challenge, and tissues were removed under aseptic conditions. Total RNA extraction from the tissues, cDNA synthesis, and qRT-PCR were performed as described above.

2.8. Cell culture

Red drum primary liver cell culture was established as described by Schmid et al. [35]. In brief, red drum liver was removed under aseptic conditions and washed three times with PBS containing 100 U of penicillin and streptomycin (Thermo Scientific HyClone, Beijing, China). The liver was cut into small pieces and digested with trypsin (Sigma, St. Louis, MO, USA). The digested solution was centrifuged at 500 × *g* for 10 min, and cell pellet was resuspended in RPMI 1640 (Thermo Scientific HyClone, Beijing, China) containing 15% foetal bovine serum (FBS) (Thermo Scientific HyClone, Beijing, China) and 100 U of penicillin and streptomycin (1640P). The cells were seeded in monolayers in 96-well culture plates with 1640P and cultivated at 25 °C.

2.9. Expression of SoFer1 in response to metal ions and H_2O_2

Red drum primary liver cells were maintained in 1640P as described above; $FeCl_2$, $CuSO_4$, and H_2O_2 (all were purchased from Sangon, Shanghai, China) were added into the cell culture at the final concentrations of 20 μM, 20 μM, and 100 μM, respectively. After incubation at 25 °C for various hours, the cells (1×10^5) were collected and used for RNA extraction with the Total RNA Kit I of Omega Bio-tek (Beijing, China). *SoFer1* expression was then determined by qRT-PCR as described above.

2.10. Plasmid construction

pET259 was constructed by inserting linker L811 (5′-TATGGCATTTAAATCTC-3′) into pET258 [32] between NdeI/XhoI sites. To construct pETRf1, the coding region of SoFer1 was amplified by PCR with primers RfrF2 (5′-GATATCATGGAGTCCCAAGTGCGTCA-3′; underlined sequence, EcoRV site) and RfrR2 (5′-GATATCGCTCTTGCCCCCCA-3′; underlined sequence, EcoRV site); the PCR products were ligated with the T–A cloning vector pBS-T (Tiangen, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the 0.5 kb fragment, which was inserted into pET259 at the SwaI site. pID3 was constructed by inserting linker EEHE (5′-AATTCGATATCCATCATCACCATCACCATTGAG-3′) at the EcoRI site of pIRES2-EGFP (Clontech, Mountain View, CA, USA). To construct pISoFer1, *SoFer1* was amplified by PCR with primers RfrF3 (5′- GATATCACCACCATGGAGTCCCAAGTGCGT-3′; underlined sequence, EcoRV site) and RfrR3 (5′-GCGATATCGCTCTTGCCCCCCAGG-3′; underlined sequence, EcoRV site); the PCR products were ligated with pBS-T, and the recombinant plasmid was digested with EcoRV to retrieve the 0.5 kb fragment, which was inserted into pID3 at the EcoRV site, resulting in pISoFer1. In pISoFer1, *Sofer1* was transcribed together with the enhanced green fluorescent protein as a single bicistronic mRNA.

2.11. Purification of recombinant protein

Escherichia coli BL21(DE3)pLysS (Tiangen, Beijing, China) was transformed with pETRf1. The transformant BL21(DE3)pLysS/pETRf1 was cultured in LB medium at 37 °C to mid-logarithmic phase, and expression of *SoFer1* was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.4 mM. After growth at 30 °C for an additional 4 h, the cells were harvested by centrifugation, and His-tagged SoFer1 was purified under native conditions using nickel–nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. To purify the bacterial protein DegQ [34], the plasmid pETQ, which expresses *degQ*, was introduced into BL21(DE3)pLysS by transformation, resulting in transformant BL21(DE3)pLysS/pETQ. Recombinant DegQ was purified from BL21(DE3)pLysS/pETQ under the same conditions as those under which recombinant SoFer1 was purified. The purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of purified protein was determined using the Bradford method [36] with bovine serum albumin as the standard.

2.12. Preparation of anti-SoFer1 antibodies

Antibodies against recombinant SoFer1 were prepared as described previously [37]. Briefly, adult rats (purchased from the Institute for Drug Control, Qingdao, China) were immunized via subcutaneous injection with recombinant SoFer1 mixed in complete Freund's adjuvant. The rats were boosted at 20, 32, and 45 days after the initial immunization. The rats were bled 14 days after the last boost, and sera were obtained from the blood. The titre and specificity of the serum antibodies were determined by enzyme-linked immunosorbent assay and Western immunoblotting analysis as described previously [38].

2.13. Cell transfection

Transient transfection of red drum primary liver cells was performed with Lipofect (Tiangen, Beijing, China) according to manufacturer's instructions. Briefly, 1 μl of Lipofect was mixed with 25 μl of RPMI 1640 without FBS and antibiotics (1640M), followed by adding into the mixture an equal volume of 1640M containing 0.5 μg of pISoFer1 or pID3. After incubation at room temperature for 20 min, the mixture was added to red drum primary liver cells in a 96-well culture plate, and the cells were incubated for 4 h at 25 °C. After the incubation, the supernatant was removed from the plate, and fresh 1640P was added. The incubation was continued for another 44 h, and the cells were examined for transfection by fluorescence microscopy, qRT-PCR, and Western immunoblotting analysis.

2.14. Protein immunoblot

pISoFer1- and pID3-transfected red drum liver cells (1×10^6) were washed several times with PBS and suspended in 50 μl of lysis buffer (7 M urea, 4% CHAPS, 2M thiourea, 60 mM dithiothreitol, 10 mM Tris, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride), followed by three times of quick freezing at liquid nitrogen and thawing at room temperature. The cells were lysed at room temperature for 2 h and then centrifuged at 13,000 *g* for 1 h. The supernatant was collected and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The proteins were subjected to SDS-PAGE, and, after electrophoresis, transferred to a nitrocellulose membrane (Amersham, Cambridge,

UK). Immunoblotting was performed as described previously [34] using anti-SoFer1 antibodies prepared above.

2.15. Cell viability analysis

The viabilities of pISoFer1- and pID3-transfected red drum liver cells were determined at different time points following transfection using the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime, Beijing, China) according to manufacturer's instructions.

2.16. Measurement of reactive oxygen species (ROS)

Red drum liver cells transfected with pISoFer1 and pID3 were maintained in 1640P at 25 °C as described above. H_2O_2 was added into the culture at various concentrations. After incubation at 25 °C for 1 h, the cells (1×10^5) were used for measurement of ROS with the Cell ROS Colorimetric Assay Kit (Genmed Scientifics, Shanghai, China) according to manufacturer's instructions.

2.17. Iron binding analysis

The iron binding activity of purified recombinant SoFer1 was determined based on the method of De Zoysa and Lee [39]. Briefly, purified recombinant SoFer1 and DegQ were dissolved in water to 6 μg/ml. Twenty microliters of 2 mM $FeCl_2$ was added into 1 ml of protein suspension or 1 ml of water (control), followed by the addition of 40 μl of 5 mM ferrozine (Sangon, Shanghai, China). After incubation at 22 °C for 10 min, absorbance at 562 nm was measured with a spectrophotometer. Iron binding activity was calculated according to the following formula: $(C - S)/C \times 100\%$, where C and S represent, respectively, absorbance values of the control and the protein under assay.

2.18. Statistical analysis

All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Statistical significances were determined with Student's t-test. In all cases, significance was defined as $P < 0.05$.

3. Results

3.1. Sequence characterization of SoFer1

The full length cDNA of SoFer1 (GenBank accession no: GU187329) is 1055 bp, which contains a 5′-UTR of 205 bp, an open reading frame (ORF) of 531 bp, and a 3′-UTR of 290 bp (Fig. 1). The 3′-UTR is followed by a putative polyadenylation signal GATAAA, which lies 12 bp upstream from the poly-A tail. The translation initiation codon (ATG) is immediately preceded by the nucleotides GCCAAA and followed by a G. These features conform well to those of the Kozak consensus sequence [40]. The ORF encodes a putative protein of 176 amino acids with a predicted molecular mass of 20.4 kDa and a theoretical isoelectric point of 5.76. BLAST analyses showed that SoFer1 shares high sequence identities (87–90%) with the ferritin M subunits of *Anoplopoma fimbria*, *Oncorhynchus mykiss*, *Salmo salar*, and *Caligus rogercresseyi*. In silico analyses identified in SoFer1 a ferritin ferroxidase center with the seven conserved residues (E24, Y31, E58, E59, H62, E104, and Q138) that are known to function as iron ligands in H/M ferritins [3,41]. The three negatively charged residues (E54, E57, and E61) that are preserved in mammalian L subunits as the nucleation site [42] are also found in SoFer1, though E57 is replaced by D57 (Fig. 1). The 5′-UTR of SoFer1 contains a putative IRE in the form of CAGTGT (Fig. 2A), which matches the ferritin IRE signature sequence CAGUGN. The putative IRE of SoFer1 has the potential to form a stem-loop structure with CAGTGT looping out at the apex (Fig. 2B). Like the archetypical ferritin IRE, SoFer1 IRE is disrupted in its hairpin structure by bulges formed by a UGC/C sequence [43].

Phylogenetic analyses showed that the vertebrate and invertebrate ferritins fell into two distinct clusters (Fig. 3). SoFer1 was placed in the vertebrate cluster, within which SoFer1 formed a subcluster with the ferritins of several fish species.

3.2. SoFer1 expression in different tissues

qRT-PCR was carried out to examine the expression profile of *SoFer1* in the gill, blood, liver, spleen, kidney, brain, heart, and muscle of red drum. The results showed that *SoFer1* expression was detected in all the examined tissues, with high levels found in liver and blood, moderate levels in muscle and spleen, and low levels in gill, brain, and heart (Fig. 4A).

3.3. SoFer1 expression in response to bacterial infection

To examine whether *SoFer1* expression was modulated by bacterial infection, red drum were experimentally challenged with the Gram-negative fish pathogen *E. tarda* and the Gram-positive fish pathogen *S. iniae*. Following infection, *SoFer1* expression in liver was analyzed by qRT-PCR at various time points. The results showed that both *E. tarda* and *S. iniae* challenges upregulated *SoFer1* expression at 4–48 h post-infection (Fig. 4B).

3.4. SoFer1 expression in response to iron, copper, and H_2O_2

To examine *SoFer1* expression under stress conditions caused by iron, copper, and oxidant challenges, primary liver cells of red drum were exposed separately to Fe(II), Cu, and H_2O_2 treatment. Subsequent qRT-PCR analyses showed that both Fe(II) and Cu exposures significantly upregulated the expression of *SoFer1* at 8 h, 12 h, and 24 h post-challenge (Fig. 5). The induction levels caused by Fe(II) were 2.5- to 3.7-fold, while those caused by Cu were 1.6- to 4.7-fold. Compared to iron and copper, H_2O_2 produced a stronger and more lasting stimulating effect on *SoFer1* expression, which was increased 7-fold at 12 h after H_2O_2 treatment.

3.5. Overexpression of SoFer1 in liver cells

To examine the effect of *SoFer1* overexpression, red drum primary liver cells were transfected transiently with the plasmid pISoFer1, which constitutively expresses *SoFer1* under the human cytomegalovirus promoter. qRT-PCR analysis showed that *SoFer1* expression in pISoFer1-transfecetd cells was 21-fold higher than that in the cells transfected with the control vector pID3. Consistently, Western immunoblot analysis showed that the amount of SoFer1 protein detected in pISoFer1-transfecetd cells at 24 h post-transfection was ~4.3-fold higher than that detected in ID3-transfecetd cells (Fig. 6). These results demonstrated that SoFer1 was indeed overexpressed and overproduced in the transfected cells.

3.6. Effect of SoFer1 overexpression on oxidative stress response

Cell viability analyses indicated that pISoFer1-transfected cells exhibited no apparent difference from pID3-transfecetd cells until 48 h post-transfection (data not shown). Since, as demonstrated above, H_2O_2 upregulated the expression of *SoFer1* in liver cells, we examined whether *SoFer1* overexpression had any effect on cellular response to H_2O_2. For this purpose, we determined the effect of H_2O_2 treatment on the production of ROS in SoFer1 transfectant at

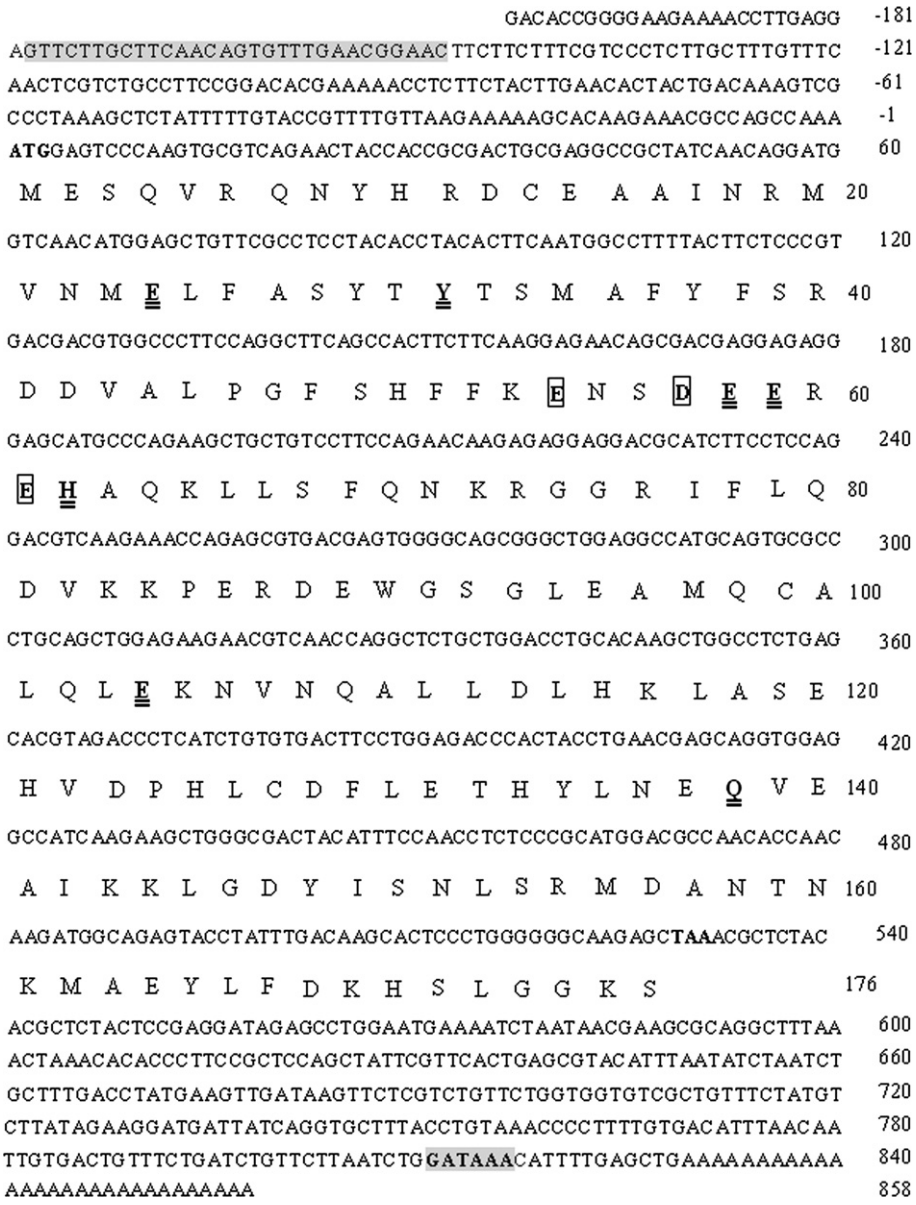

Fig. 1. The nucleotide and deduced amino acid sequences of SoFer1. The nucleotides and amino acids are numbered along the right margin. The iron response element (IRE) sequence is in grey, the translation start (ATG) and stop (TAA) codons are in bold, the seven amino acids involved in iron binding are in bold and doubly underlined, the three amino acids involved in iron nucleation are in bold and boxed, and the putative polyadenylation signal (GATAAA) is in bold and grey.

24 h post-transfection. The results showed that H_2O_2 exposure caused a dose-dependent increase of ROS in both SoFer1 transfectant and the control cells (Fig. 7); however, the amounts of ROS detected in SoFer1 transfectant were much lower than those detected in the control cells, especially when the challenging H_2O_2 was at higher doses.

3.7. Expression and purification of recombinant SoFer1

In order to examine the biological property of SoFer1 in the form of recombinant protein, the coding sequence of SoFer1 was subcloned into *E. coli*, and recombinant SoFer1 was purified under native conditions to maintain its natural structure and biological property. SDS-PAGE analysis showed that the purified protein exhibited a molecular mass comparable to that predicted for recombinant SoFer1 (21.3 kDa) (Fig. 8).

3.8. Iron binding activity of purified recombinant SoFer1

To examine whether SoFer1 possessed the fundamental property of ferritin, which is iron binding, purified recombinant SoFer1 was subjected to iron-chelating assay. The results showed that the assay solution containing SoFer1 exhibited an A_{562} absorbance of 0.32, while the control solution exhibited an A_{562} absorbance of 0.87, which correspond to an iron binding activity of 63.2% for SoFer1. In contrast, recombinant DegQ, a protein with no iron-chelating capacity and purified under the same conditions as those under which SoFer1 was purified, exhibited no detectable iron-chelating activity.

4. Discussion

In this report, we described the identification and analysis of a ferritin subunit, SoFer1, from red drum. SoFer1 shares high

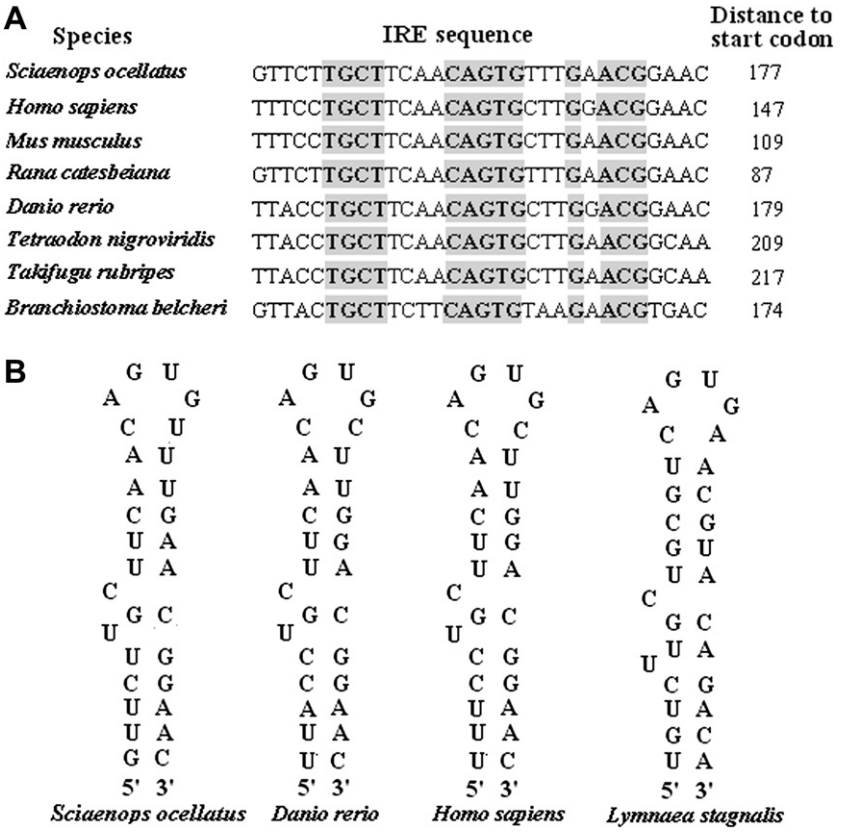

Fig. 2. Comparison of the iron response element (IRE) and stem-loop structures of ferritins from different species. (A) Alignment of the SoFer1 IRE (*Sciaenops ocellatus*) with IREs of ferritins from a number of species. Consensus nucleotides are shaded. (B) Predicted IRE stem-loop structures of the ferritins from *Sciaenops ocellatus*, *Danio rerio*, *Homo sapiens*, and *Lymnaea stagnalis*.

sequence identities with a number of known M ferritins and possesses sequence features that distinguish M ferritin from H and L ferritins. Of the several fish ferritins that have been studied at transcription level, H ferritins are known to express mainly in liver and blood [12,44,45], while M ferritins are predominately found in spleen and liver [15]. In the case of SoFer1, we found that its expression was relatively high in liver and blood and moderate in spleen. Since no H ferritin has been identified in red drum, we could not determine the expression level of *SoFer1* relative to that of H subunit. However, given the fact that liver and blood play essential roles in iron metabolism and storage and that SoFer1 is highly expressed in these tissues, it is reasonable to speculate that SoFer1 is a functional ferritin that participates in iron homeostasis in red drum.

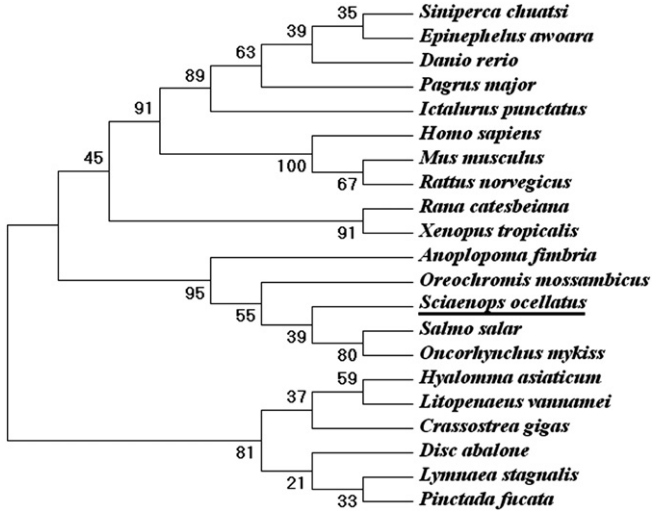

Fig. 3. Phylogenetic tree of ferritin. The phylogenetic tree was constructed using the neighbor-joining method based on the deduced amino acid sequences of ferritins from a number of species. Numbers on the nodes indicate the percentage frequencies in 1000 bootstrap replications.

Studies of human and murine ferritins have shown that iron modulates ferritin synthesis at both transcription and post-transcription levels. At the transcription level, prolonged iron exposure was found to increase the expression of L subunit but had no effect on H subunit [20,46,47]. However, treatment of Hela cells with iron augmented the mRNA levels of both H and L subunits [19]. In our study, we found that iron treatment caused significant induction of *SoFer1* expression in red drum liver cells. The induction levels, 2.5- to 3.7-fold, were comparable to those reported for mammalian L subunits. This observation, together with the fact that SoFer1 resembles L ferritin in sequence features, favors the hypothesis that, in red drum, SoFer1 may play a role analogous to that played by L ferritin in mammals. Since SoFer1 possesses an IRE sequence at the 5′-UTR, it is possible that SoFer1 synthesis may also be modulated by iron at the translation level through the actions of IRP-like proteins.

Copper as a ferritin regulator has been observed in *Xenopus laevis* cells [48] and Chinese shrimp (*Fenneropenaeus chinensis*) [21]. In a recent study by Prieto-Alamo et al. [49], it was found that in the fish *Solea senegalensis*, M ferritin expression was upregulated by $CuSO_4$ in both liver and kidney. Similarly, in our study, we found that significant induction of *SoFer1* expression occurred following

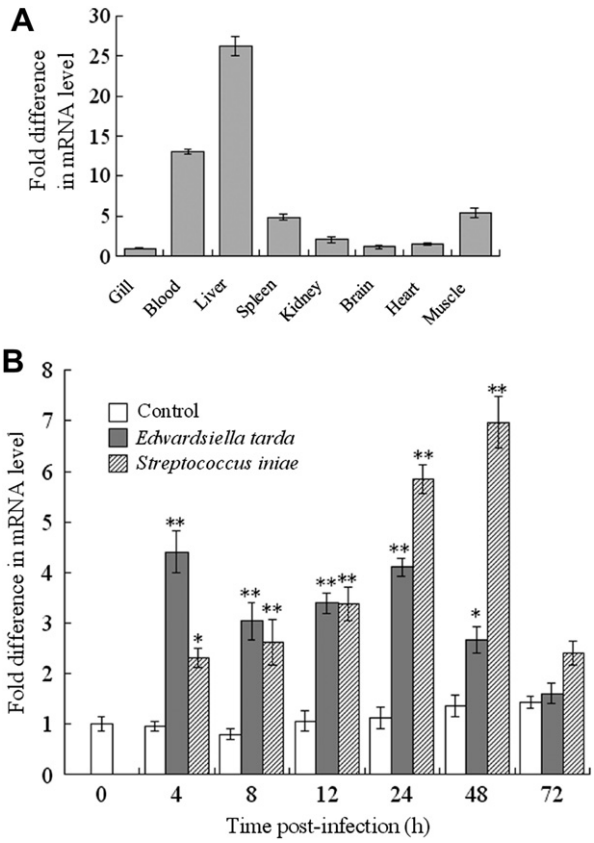

Fig. 4. *SoFer1* expression in red drum tissues and in response to bacterial challenge. (A) *SoFer1* expression in different tissues of red drum detected by quantitative real time reverse transcriptase PCR (qRT-PCR). *SoFer1* expression levels in blood, liver, spleen, kidney, brain, heart, and muscle are normalized to that of gill. Vertical bars represent means ± SE ($N = 5$). (B) Red drum were challenged with *Edwardsiella tarda*, *Streptococcus iniae*, or PBS (control). *SoFer1* expression in liver was determined by qRT-PCR at various times post-challenge. The mRNA level of SoFer1 was normalized to that of β-actin. Values are shown as means ± SE ($N = 5$). Significances between PBS-challenged fish and bacterium-challenged fish are indicated with asterisks. **, $P < 0.001$; *, $P < 0.05$.

copper exposure. It is of note that the *SoFer1* expression pattern induced by copper bears a general resemblance to that induced by iron, which tends to suggest that the signal transduction pathways elicited by the two metals may share some common aspects. Like

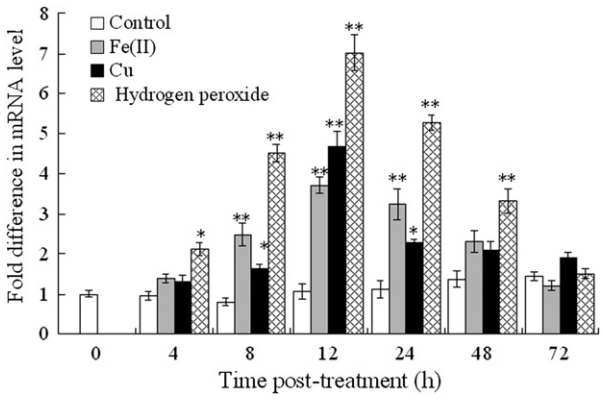

Fig. 5. *SoFer1* expression in red drum liver cells in response to iron, copper, and hydrogen peroxide. Red drum liver cells were treated with $FeCl_2$, $CuSO_4$, and H_2O_2, respectively, and *SoFer1* expression was determined by quantitative real time reverse transcriptase PCR at various times post-treatment. The mRNA level of SoFer1 was normalized to that of β-actin. Values are shown as means ± SE ($N = 5$). Significances between control (untreated cells) and metal ions/H_2O_2-treated cells are indicated with asterisks. **, $P < 0.001$; *, $P < 0.05$.

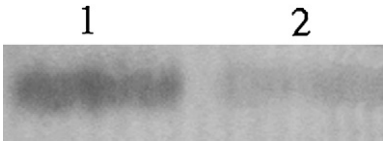

Fig. 6. Western immunoblot analysis of SoFer1 production in pISoFer1- and pID3-transfected cells. Proteins were prepared from equal amounts of pISoFer1-transfected (lane 1) and pID3-transfected (lane 2) red drum liver cells and resolved by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane and blotted with anti-SoFer1 antibodies.

iron, copper is an essential metal ion required by most living organisms; however, again like iron, excess copper is toxic to the cell on the account that copper, through its ability to catalyze certain redox reactions in the presence of oxygen species, can lead to the generation of free radicals. It therefore stands to reason that enhanced production of ferritin may serve as a protective mechanism to detoxify the effect of copper.

Among the modulators of ferritin synthesis, inducers of oxidative response are known to have a potent and general effect on both the transcription and translation of ferritin [28,50]. Studies of Hela cells and murine erythroleukemia cells indicated that overexpression of ferritin H reduced cellular levels of ROS and enhanced the ability of the cells to cope with oxidative stress [51,52], whereas interference with ferritin H expression sensitized cells to oxidative damage [53]. In Hela cells, it was also observed that overexpression of ferritin L affected ROS production and resistance against oxidative stress [1]. In our study, we found that treatment with the strong oxidizer H_2O_2 significantly upregulated *SoFer1* expression, which was in magnitude much higher than those induced by iron and copper. In agreement with this observation, compared to control cells, cells that overexpressed *SoFer1* produced much lower levels of ROS following H_2O_2 treatment, especially when the dose of H_2O_2 was increased to higher levels. Hence, SoFer1 seemed to exert an attenuating effect on the production of ROS, and this effect became more apparent as the severity of the oxidative stress encountered by the cell increased. These results support the idea that SoFer1 is involved in anti-oxidative damage.

Many studies have demonstrated that ferritin synthesis is affected by inflammatory conditions, including those caused by bacterial infections [54]. Compared with mammalian ferritins, fish ferritins are much less studied in this respect. Of the few documentations that are available, it is known that the expression of the H ferritins of Atlantic cod and channel catfish was augmented by

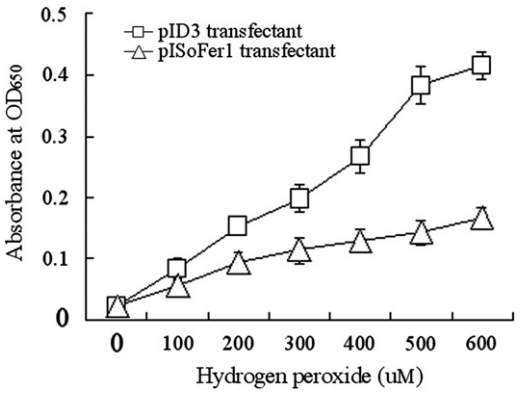

Fig. 7. Effect of *SoFer1* overexpression on the production of reactive oxygen species (ROS) in red drum liver cells. Red drum liver cells transfected with pISoFer1 and pID3 were treated with different doses of hydrogen peroxide, and production of ROS was measured as absorbance at OD_{650}.

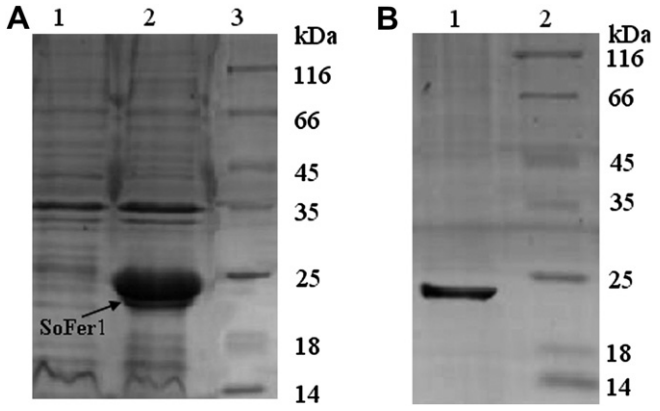

Fig. 8. SDS-PAGE analysis of recombinant SoFer1. (A) Whole cell proteins were prepared from *E. coli* BL21(DE3)pLysS/pETRf1 cultured in LB medium before (lane 1) and after (lane 2) isopropyl-β-D-thiogalactopyranoside induction. The arrow indicates the protein band representing SoFer1. Lane 3, protein markers. (B) Recombinant SoFer1 after purification by affinity chromatography. Lane 2, protein markers. In both panels, the proteins were resolved by SDS-PAGE and viewed after staining with Coomassie brilliant blue R-250.

challenging with the bacterial pathogens *Aeromonas salmonicida* and *Edwardsiella ictaluri*, respectively [55,56]. Likewise, the expression of the sea bass H ferritin was upregulated upon exposure to *Photobacterium damselae* spp. *Piscicida* [44]. Similar to these previous observations, we found that *SoFer1* expression was elevated by exposure to fish pathogens of both Gram-positive and Gram-negative natures. Since iron is an essential nutrient for most bacterial pathogens, the upregulation of *SoFer1* may, like what have been observed with mammalian ferritins under conditions of clinical infection, constitute a defence mechanism against bacterial infection by depriving free iron from the cellular environment.

In conclusion, the results of this study demonstrate that SoFer1 is an M ferritin homologue whose expression was modulated by iron, copper, oxidant, and bacterial infection. These observations, together with the fact that recombinant SoFer1 possessed iron binding capacity, suggest that it is likely that in red drum, SoFer1 functions as a biologically active ferritin and participates in iron storage, anti-bacterial infection, and alleviation of oxidative stress.

Acknowledgements

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