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Identification of multiple ferritin genes in *Macrobrachium nipponense* and their involvement in redox homeostasis and innate immunity

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ABSTRACT

Based on the transcriptome database, we screened out four ferritin subunit genes (MnFer2-5) from the oriental river prawn *Macrobrachium nipponense*, which encode two non-secretory and two secretory peptides. MnFer2 and 4 possess a strictly conserved ferroxidase site, and MnFer3 has a non-typical ferroxidase site. MnFer5 seems to be a number of ferritin families, which has a distinct dinuclear metal binding motif, but lacks an iron ion channel, a ferroxidase site and a nucleation site. Diverse tissue-specific transcriptions of the four genes indicate their functional diversity in the prawn. Among them, MnFer2 is mainly expressed in hepatopancreas and intestines, MnFer3 and 4 are predominantly expressed in gills, and MnFer5 is widely expressed in various tissues with high presence in intestines, hepatopancreas and haemocytes. The transcription of all the four MnFer genes can be strongly induced by doxorubicin, indicating the involvement of these ferritin subunits in protection from oxidative stress. Upon *Aeromonas hydrophila* infection, only MnFer5 is persistently up-regulated, while other subunits including MnFer2-4 are down-regulated during the early stage, followed by recovery and even a slight increase at 48 h post bacterial challenge. Moreover, the iron binding capacity of recombinant MnFer2 is also demonstrated in vitro. The *E. coli* expressing MnFer2 displays increased resistance to hydrogen peroxidase cytotoxicity. These results suggest a protective role of ferritins from *M. nipponense* in iron homeostasis, redox biology and antibacterial immunity and shed light on the molecule evolution of crustacean ferritin subunits.

1. Introduction

Iron is an essential trace element in all living organisms and involved in multiple metabolic processes, including respiration ((Fe−S)-containing ferredoxins, heme-contained cytochromes) and key enzymatic reactions (e.g. (Fe−S) proteins, such as the fumarase and aconitase of the TCA cycle) [1]. Even so, excess iron potentiates oxygen toxicity via Fenton reaction, converting the less reactive hydrogen peroxide (H_2O_2) to the more harmful reactive oxygen species (ROS), such as the highly destructive hydroxyl radical (·OH) [2]. The strict control of iron homeostasis is associated with defenses against oxidative stress in vivo. A number of specialized iron-binding proteins have evolved to keep iron in a safe and bioavailable form. Transferrin and ferritin have attracted a plenty of attention due to their important roles in iron transport and storage. Transferrin is a glycoprotein found in multicellular animals and characterized with high affinity for iron and ability of iron transportation. Ferritins are found throughout animals, plants, and microbial kingdoms, and are characterized by high capacity for iron storage [3]. Ferritins can store excess iron and play an important role in iron homeostasis and management of oxidative stress response in invertebrates [4].

Members of the ferritin family in most mammals and insects are generally composed of two kinds of subunits: heavy chain homologs (HCHs) and light chain homologs (LCHs). With a conserved ferroxidase site, HCHs can facilitate the rapid oxidation and uptake of ferrous iron. LCHs lacks the ferroxidase site, but it can facilitate the nucleation of the ferrihydrite iron core [5,6]. The M (middle) type ferritin subunit, another type of subunit, is found in some vertebrates such as fish. It possesses both the ferroxidase di-iron center and the ferrihydrite nucleation center [7,8]. In contrast to the predominant cytosolic location of mammalian ferritins serving as iron storage proteins, most insect ferritins are secretory and play roles in iron transport [9,10]. In addition to the well-characterized cytoplasmic and serum ferritin, a special mitochondrial ferritin has been identified in some mammals and invertebrates such as *Drosophila,* which limits oxidative damage regulating mitochondrial iron availability [11–13].

The molecular characterization and functions in iron mechanism and antioxidant response of mammalian ferritins have been heavily

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investigated in past several decades. In contrast, very few studies have explored on their invertebrates, especially crustacean counterparts. Phylogenetic and structural analysis have revealed unique features of crustacean ferritins [14]. Ferritins have identified and characterized a few crustacean species including *Litopenaeus vannamei* [15,16], *Macrobrachium rosenbergii* [17], *Penaeus monodon* [18], *Fenneropenaeus chinensis* [19], *Exopalaemon carinicauda* [20], *Marsupenaeus japonicas* [21], *Eriocheir sinensis* [22], and *Macrobrachium nipponense* [23]. Results of gene expression analysis hint that crustacean ferritins are involved in innate immunity against pathogens (e.g. bacteria, LPS and virus) [16,21–24], and resistance to various stress including pH, heavy metal challenge, hypoxia, desiccation and low salinity [19,20,24–27].

The oriental river prawn *Macrobrachium nipponense* (Decapoda; Palaemonidae) is a typical freshwater crustacean species widely distributing in brackish and fresh waters throughout China and other Asian countries [28]. *M. nipponense* is also an important commercial fishery resource for aquaculture in China with a cultured production of about 272,592 tons in 2016 [29]. The need for healthy breeding has led us to pay attention to the innate immune mechanism of the freshwater prawn. In the previous study, Sun et al. cloned a ferritin gene encoding a protein, named MnFer (accession no. in GenBank KC825355), with iron binding capacity from *M. nipponense*. In the present study, 4 new ferritin isoform transcripts, which were designated as MnFer2-5 following by the previous study MnFer (as MnFer1), both non-secretory and secretory, were identified through screening the transcriptome database of *M. nipponense*. Different tissue distribution patterns and distinct expression profiles responding to the bacterial challenge were detected among the four isoforms.

2. Materials and methods

2.1. Prawns and tissue sampling

Wild healthy prawns, *M. nipponense*, with average body weight of approximately 3.5 g, were obtained from the Baiyang Lake in Hebei Province, China. The prawns were cultured in 50-L tanks with aerated freshwater at 22 ± 1 °C and fed with artificial bait twice a day for one week prior to experimentation in the laboratory. Hemolymph was drawn from the healthy prawns in an equal volume of anticoagulant (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7) and haemocytes were immediately isolated by centrifugation at 800*g* for 10 min. Meanwhile, intestines, muscles, gills, and hepatopancreas were dissected out from the prawns. All the haemocytes and tissues were preserved in liquid nitrogen for further detection of gene expression.

2.2. Oxidative stress and immune challenge

For oxidative stress trials, prawns were injected with a free radical-generating agent, doxorubicin (DOX), which is believed to disrupt redox balance in cells by interfering with the mitochondrial phospholipid cardiolipin and causing the production of ROS [30]. 60 accommodated prawns were randomly divided into 6 groups with 10 individuals in each group. They were injected in the abdominal muscle with about 20 μl DOX solution, and the doses are 0, 0.5, 1.25, 2.5, 5, and 10 μg/g body weight. 6 individuals from each group were sampled and their hepatopancreas were ablated at 12 h post injection for qRT-PCR analysis and ROS and MDA concentration measurement. The immune challenge trial was conducted according to the previous description [31]. Generally, each prawn was injected with a 20-ml suspension of live *Aeromonas hydrophila* (2×10^7 CFU) in physiologic saline solution into the abdominal muscle. The hepatopancreas and gills from six live individuals were randomly sampled at 0, 6, 12, 24, and 48 h post injection for qRT-PCR analysis.

Table 1
Primers used in this study.

Use of primers	Primer name	Primer sequence
cDNA cloning		
	AOLP	GGCCACGCGTCGACTAGTACT$_{16}$ (G/A/C)
	MnFer2-F1	ACAAGCTGCTCCGCGTC
	MnFer2-R1	GTCAGTGAATCCTCCCTGTAG
	MnFer3-F1	CATCGTAGGTTGGTGGC
	MnFer3-R1	GGTGCTTCTAGGCACAAG
	MnFer4-F1	GGCAGTAGAATACGGACATC
	MnFer4-R1	CATGACATCAAGGCAGTGC
	MnFer5-F1	ATGATTGCCAGGGCAATG
	MnFer5-R1	TTACAGATCAGAGTCAAAGATATG
Real time PCR		
	Actin-F	TGACCACTGCCGCCTCCTC
	Actin-R	TGCCGCAAGATTCCATACCC
	MnFer2-F2	GCTACTACGCCAGGGATGA
	MnFer2-R2	CAGGAAGTCGCAGAGGTGT
	MnFer3-F2	CAGAATGATGCTCTTCCCAATG
	MnFer3-R2	AGACCAAAGCCAGACCCAAC
	MnFer4-F2	GACTACCTGAATCTCCGTGGC
	MnFer4-R2	ACCGTCGTGCTGTTCGTC
	MnFer5-F2	AGCCGTCAGTGGGAAGAAG
	MnFer5-R2	TTGGAGTCATCCAGCATCG
Recombinant expression		
	MnFer2-F3	CGGGATCCATGGCCAGCAAGATCCGC
	MnFer2-R3	CCCAAGCTTTTACTGGAGTGATTTGTCGAAG

2.3. RNA extraction and reverse-transcription

Total RNA was extracted from haemocytes and various tissues with the Trizol reagent (Invitrogen) following the manufacturer's protocol and treated with RQ1 RNase-Free DNase (Promega) to remove the contaminated DNA. cDNA was reversely transcripted from total RNA by M-MLV reverse transcriptase (Promega) following the manufacturer's protocol with a universal primer AOLP (Table 1).

2.4. Transcriptome-wide screening and sequence validation

Four ferritin protein sequences of *Macrobrachium* (three of *M. rosenbergii* and one of *M. nipponens*) were collected from the UniProtKB/Swiss-Prot and used to query the *M. nipponense* transcriptome database of 7 different tissues (SRX142691) [32] in NCBI by a tBLASTn program at CLC Main Workbench 5.5 with a cutoff E-value of 10^{-5}. The corresponding transcript sequences of the hit genes were further verified by searching the NCBI nr database using BLASTx. For sequence validation, primers covering the full-length open reading frame (ORF) of each gene (Table 1) were used to amplify each gene using the cDNA of hepatopancreas or gills as a template.

2.5. Sequence analysis

The deduced amino acid sequences were obtained using an ORF finder program (http://ncbi.nlm.nih.gov/gorf/gorf.html) and then analyzed with the Expert Protein Analysis System (http://www.expasy.org). The potential signal peptide was predicted through SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The protein domains were searched via web CD-search tools, including SMART for Ensembl database (http://smart.embl-heidelberg.de) and Batch for NCBI database (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). The sub-cellular location of each gene was predicted through TargetP (http://www.cbs.dtu.dk/services/TargetP/) and CELLO (http://cello.life.nctu.edu.tw/). The potential iron response element (IRE) for the mRNA of each gene was predicted through SIREs Web Server (http://ccbg.imppc.org/sires/index.html). The homology search of the Nr database was performed using the BLASTp algorithm at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignments were carried out with the CLC Main Workbench software. A phylogenetic

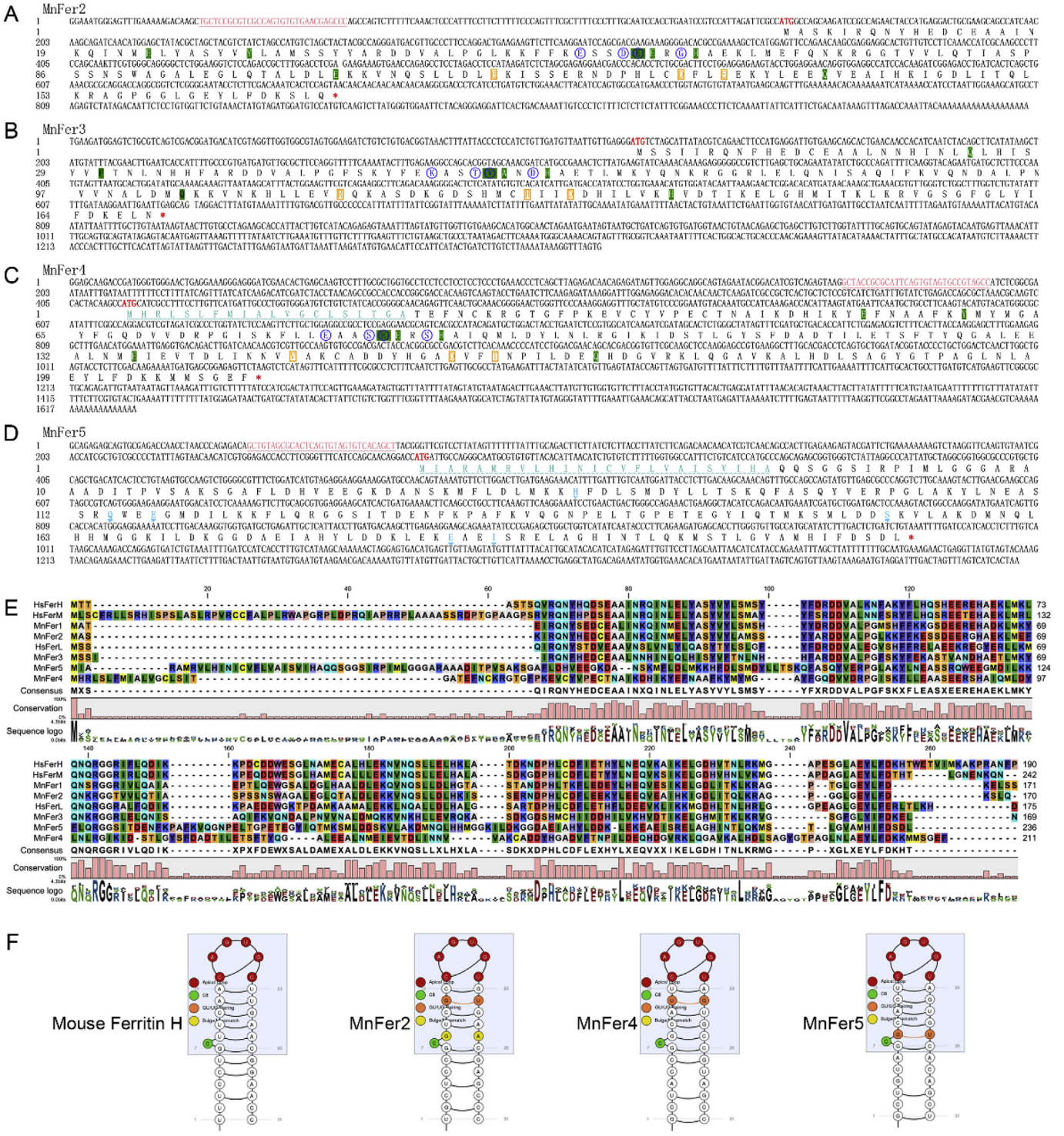

Fig. 1. The nucleotide and deduced amino acid sequences of MnFer2-5 (A–D). The IRE with the signature sequence (5′-CAGTGN-3′) and the bulged cysteine at 6 nt upstream of the IRE loop in the 5′UTR are underlined. The start codon is marked in bold and the stop codon is indicated using "*". The predicted signal peptides are waved lines. The eight or seven residues associated with the iron binding site are shaded. The four conservative residues involved in the ferrihydrite nucleation center are circled. The three key residues that make up the iron ion channel are boxed. The six residues related to the dinuclear metal binding motif of MnFer5 are doubly underlined. Alignment of the amino acid sequence of MnFers with the *Homo sapiens* ferritins (E). The stem-loop structures of Mouse Ferritin H (NM_010239), MnFer2, MnFer4 and MnFer5 (F).

tree was constructed using amino acid sequences of ferritin subunits from *Macrobrachium* and representative animals in different phyla, including vertebrates, arthropods and mollusks by the Neighbor-joining (NJ) method. One thousand bootstraps were performed to assess nodal support.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Expression levels of mRNA for MnFers were measured by qRT-PCR on a LightCycler system (Roche) using the SYBR Green kit (TaKaRa, China). The primer sequences used for amplifying target genes and β-

Table 2
The predicted properties of MnFers.

MnFers	MnFer1	MnFer2	MnFer3	MnFer4	MnFer5
Accession no. in GenBank	KC825355	MK173047	MK173048	MK173049	MK173050
Amino acid number	171	170	169	211	236
Length of signal peptide (Amino acid number)	N	N	N	20	25
Calculated molecular mass of mature peptide (kDa)	19.40	19.20	19.35	21.34	23.30
Theoretical isoelectric point (pI)	4.86	5.27	7.87	4.70	5.51
Sub-cellular location	Cytoplasmic	Cytoplasmic	Nuclear	Extracellular	Extracellular
Domain of Ferritin-like superfamily	Y	Y	Y	Y	Y
Ferroxidase di-iron center (ion binding site)	E24, Y31, E58, E59, E61, H62, E104, Q138	E24, Y31, E58, E59, H62, E104, Q138	Q24, F31, V58, A59, H62, Q104, H138	E52, Y59, E86, E87, H90, E136, Q168	N
Ferrihydrite nucleation center	K54, D57, E58, E61	E54, D57, E58, G61	K54, T57, V58, D61	E82, S85, E86, S89	N
Iron ion channel	H115, K128, E131	H115, D128, E131	R115, H128, D131	V147, D158, T161	N
Dinuclear metal binding motif	N	N	N	N	H79, Q114, E117, S168, E205, I208
Iron-responsive element (IRE)	N	Y	N	Y	Y

actin genes as internal control were listed in Table 1. Expression levels of the target genes were calculated by comparing the cycle threshold value (Ct) with the reference gene β-actin. The relative mRNA expression was calculated using the △△Ct method [33]. All the results were generated with three technical replicates. The significance at $p < 0.05$ was analyzed using one-way ANOVA.

2.7. Recombinant expression, purification and renaturation of MnFer2

The DNA fragment encoding MnFer2 was obtained by PCR with a pair of primers MnFer2-exF and MnFer2-exR with restriction enzyme sites for *Bam*H I and *Hind* III at the 5′-end. Both the amplified fragment and the expression vector pET-30a were digested with *Bam*H I and *Hind* III, and ligated together with T4 DNA ligases. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) for expression. After being induced by 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C for 4 h, the cells were harvested by centrifugation and the recombinant MnFer2 protein was purified using Ni-NTA Resin (GenScript, China). The purified protein was refolded in a gradient urea-TBS glycerol buffer (50 mM Tris–HCl, 50 mM NaCl, 10% glycerol, 2 mM reduced glutathione, 0.2 mM oxide glutathione, a gradient urea concentration of 6, 5, 4, 3, 2, 1, 0 M urea, pH 8.0; each gradient at 4 °C for 12 h) [34]. The resultant proteins were separated by reducing 12% SDS-PAGE, and visualized with Coomassie bright blue R250. The concentration of purified rMnFer2 was quantified by the Bradford method.

2.8. Iron binding assay of rMnFer2

The iron binding activity of refolded rMnFer2 was analyzed according to a reported protocol [35]. Generally, the purified rMnFer2 and bovine serum albumin (BSA) as control were dissolved in water at different concentrations of 10, 5, 2.5 and 1.25 μg/ml. Then 20 μl of 2-mM $FeCl_2$ was added into 1 ml rMnFer2 or BSA suspension, followed by the addition of 40 μl of 5 mM ferrozine (Sangon, Shanghai, China). After incubation at 22 °C for 10 min, the absorbance at OD_{562} was measured with a spectrophotometer. Three replicates were performed for each assay and the average absorbance values were used for further analysis. Iron binding activity was calculated according to the following formula: (C−S)/C × 100%, where C and S represent the absorbance values of the control and the protein, respectively.

2.9. H_2O_2 tolerance assay of MnFer2

The antioxidant function of MnFer2 in vivo was further investigated via H_2O_2 tolerance assay according to the method described by Yu et al. [36]. Briefly, both *E. coli* expressing MnFer2 and strain containing empty vector pET30a were cultured in LB medium with kanamycin at 37 °C till OD_{600} reaches 0.6. Protein expression was then induced for 1 h with IPTG at final concentration of 0.1 mM at 28 °C. Then the cells were diluted into equal densities (OD) at 600 nm 200 μl cells expressing MnFer2 or not were mixed with 200 μl H_2O_2 at concentrations of 0, 30 and 50 mM and were incubated with gentle shaking at 28 °C for 1 h. Subsequently, a 10 μl droplet of each trial was plated on LB medium and incubated overnight at 37 °C. Bacterial growth was observed and recorded. All the experiments were repeated three times.

3. Results

3.1. cDNA cloning and sequence analysis of Mnfers

In addition to the previously reported MnFer, here referred to as MnFer1, four unique ferritin subunit transcripts were screened out from the transcriptome database of *M. nipponense*. These sequences were amplified and sequenced for validation and then designated as MnFer2-MnFer5. Putative iron responsive elements (IREs), with the conserved 5′-CAGUGU-3′ loop at the top in the 5′-UTR of MnFer2 (Fig. 1A), MnFer4 (Fig. 1C) and MnFer5 (Figure, 1D) mRNAs were observed. However, IRE was absent in MnFer1 and MnFer3 (Fig. 1B) mRNAs. The molecular characters of these speculated peptides were showed in Table 2. The four MnFers consist of 170, 169, 211 and 236 amino acids respectively, two (MnFer4 and MnFer5) of which have a putative signal peptide, hinting a secretory expression. Speculated pI of the mature peptides declared an identical anion property of MnFer2, 4 and 5 subunits with pI at 5.27, 4.85 and 5.84. As an exception, the pI of MnFer3 is 7.87. Conserved domain prediction using the SMART software and Web CD-Search Tool indicated that all the four subunits (MnFer2-5) contain a Ferritin-like superfamily domain. We then checked the conserved amino acid residues that might comprise the three key domains of the ferritin subunits, including an iron ion channel and a ferroxidase di-iron center that are present in H type subunits, as well as the ferrihydrite nucleation center typically observed in L type subunits (Table 2). Seven conserved residues (E28, Y35, E62, E63, E65, H66, E108, Q142 by human H-type ferritin numbering) in the ferroxidase centers responsible for oxidation of Fe (II) in vertebrate H-type ferritin are found in MnFer2 (E24, Y31, E58, E59, H62, E104, Q138) and MnFer4 (E52, Y59, E86, E87, H90, E136, Q168). While the corresponding residues in MnFer3 are Q24, F31, V58, A59, H62, Q104 and H138 (Fig. 1 and Table 2), which are harbored in a putative ferroxidase center predicted by Web CD-Search Tool. On the other hand, four acidic amino acid residues of the putative nucleation site, which correspond respectively to E57, E60, E61, and E64 of the human L-type ferritin, are present in the four subunits of MnFer2-4 (Fig. 1 and Table 2). In

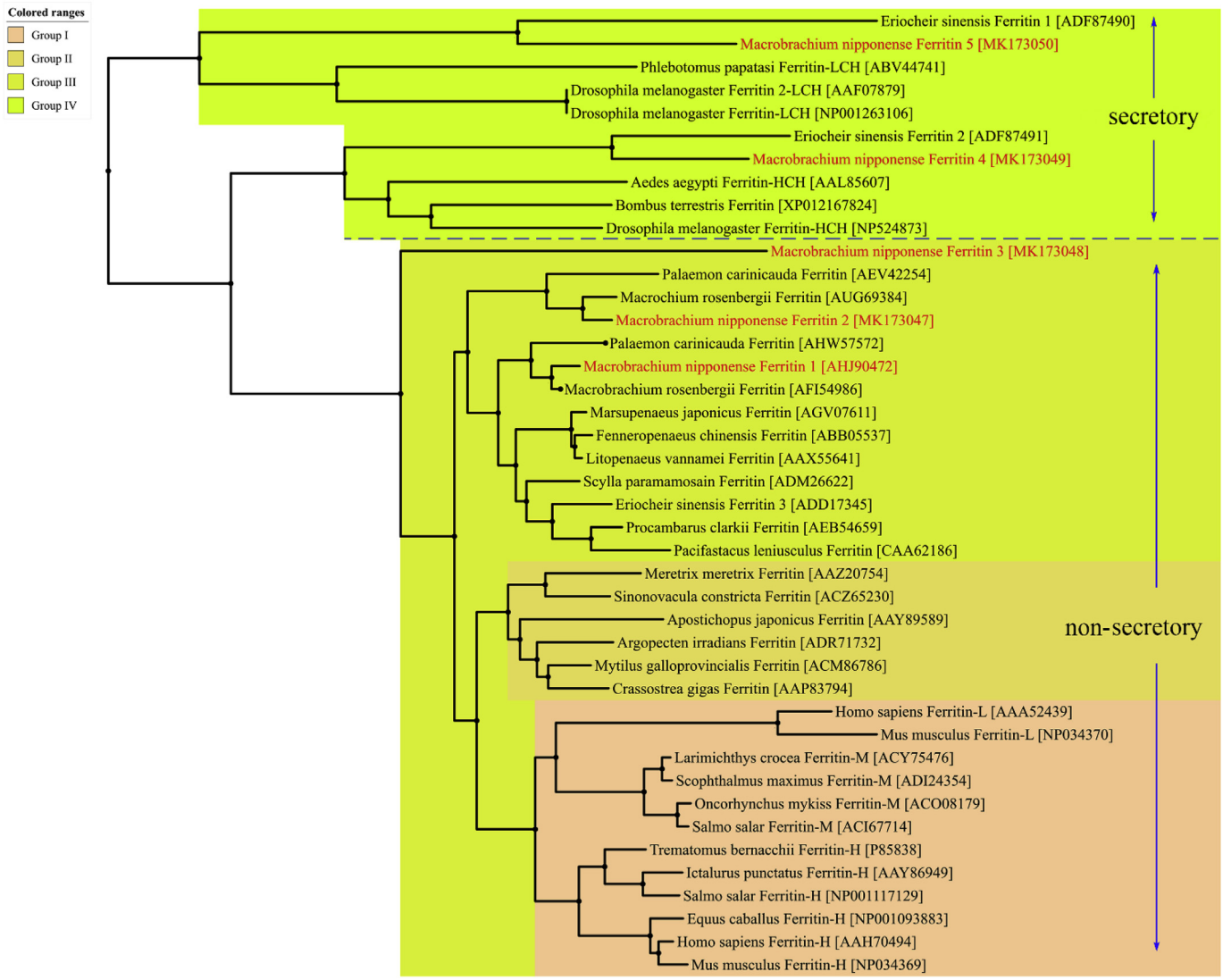

Fig. 2. Neighbor-Joining (NJ) algorithm phylogenetic tree based on amino acid sequences of ferritins from *Macrobrachium nipponense* and other representative species of crustaceans, insects, mollusks and vertebrates. The tree is clearly divided into two main clades of non-secretory and secretory ferritin subunits.

contrast, MnFer5 contains a predicted Ferritin-like domain, but iron ion channels, ferroxidase sites, and nucleation sites appeared to be absent in this molecule. However, a distinct dinuclear metal binding motif (H79, Q114, E117, S168, E205, I208) was unexpectedly found in MnFer5 (Table 2). MnFer1-5 protein sequences were used to predict the subcellular location with available online tools CELLO and TargetP programs. The results suggested that MnFer1and MnFer2 located in cytoplasm, MnFer3 located in cell nucleus, MnFer4 and MnFer5 were secretory extracellular proteins.

3.2. Homologous and phylogenetic analysis of MnFers

Homology analysis indicated that MnFer2-5 respectively shared the greatest identity with ferritins from *Macrobrachium rosenbergii* (91%), *Marsupenaeus japonicus* (51%), *Eriocheir sinensis* (54%), and *Rimicaris exoculata* (60%). An unrooted phylogenetic tree was constructed using MnFer1-5 and 37 representative ferritin sequences from vertebrates and invertebrates. In the phylogenetic tree, two main clades of non-secretory (Group I ~ III) and secretory subunits (Group IV) can be clearly identified (Fig. 2). MnFer1, MnFer2 and MnFer3 were gathered with the vertebrate non-secretory ferritins, while MnFer4 and MnFer5 fit in the group with arthropod secretory ferritins. For the non-secretory clade, three subgroups representing vertebrate, molluscan and crustacean ferritins were clearly distinguishable. However, the predicted nuclear MnFer3 floated outside these three subgroups as an outgroup. MnFer5, a number of ferritin superfamilies without iron ion channels, ferroxidase sites, or nucleation sites appeared to represent a more ancient form basing on the phylogenetic tree.

3.3. Tissue distribution of MnFers mRNA

Transcription levels of MnFer2-5 in different tissues were investigated by qRT-PCR with β-actin as internal control (Fig. 3A). The mRNA of MnFer2 was mainly expressed in hepatopancreas, slightly expressed in intestines, and scarcely expressed in other tissues. MnFer3 mRNA was widely distributed throughout the tested tissues with the highest level in gills. MnFer4 predominantly was expressed in gill, moderately expressed in muscle and hardly expressed in other tissues including hepatopancreas, intestines and haemocytes. MnFer5 showed robust expression in all detected tissues, in particular in intestines and hepatopancreas. Diversity of tissue distribution of these subunits indicated functional differences.

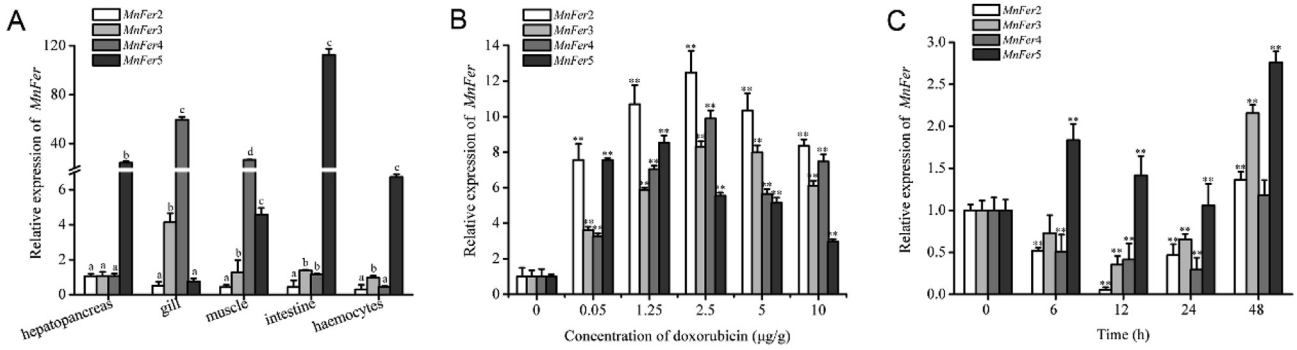

Fig. 3. (A) MnFer2-5 mRNA expression levels in various tissues of *Macrobrachium nipponense* detected by qRT-PCR. (B) Expression profiles of MnFer2 and 5 in hepatopancreas, and MnFer3 and 4 in gills under doxorubicin injection and (C) bacterial challenge. Vertical bars represent the mean ± SE (N = 6) and bars with different letters were significantly different ($p < 0.05$). Asterisks indicate significant differences (**, $p < 0.01$) versus the unchallenged samples.

3.4. Expression profiles of MnFers mRNA under oxidative stress and immune challenge

The relative expression levels of MnFer2-5 in their respective main tissues of existence, the hepatopancreas and gills, were analyzed after DOX or bacteria challenge using qRT-PCR. Compared with controls, significant up-regulations of all the MnFers transcripts were recorded in prawns at 12 h post DOX injection in a dose-dependent manner (Fig. 3B). The highest expression levels consistently presented when the MnFer2 was challenged by DOX at 2.5 μg/g body weight. After bacteria challenge, similar expression profiles of the MnFer2-4 subunits were detected: significant and sustaining decreases appeared during the early stage (6–24 h), followed by recovery and even a slight increase at 48 h post injection. In contrast, the expression of MnFer5 mRNA significantly increased at each monitoring point post bacteria injection and the expression reached the maximum value at 48 h post-challenge (Fig. 3C).

3.5. Expression and iron binding activity of MnFer2

To investigate the biological function of MnFers, the hepatopancreas cytoplasmic MnFer2 was recombinantly expressed in *E. coli* as a representative, and rMnFer2 was purified from the cell lysate under a denatured condition. As the SDS-PAGE analysis in Fig. 4A showed, the purified protein exhibited a molecular mass comparable to that predicted for MnFer2 (21 kDa). Then the purified protein was subjected to a strict renaturation process. After that, the iron binding activity was detected. As shown in Fig. 4B, with an increasing concentration of the rMnFer2, the absorbance values at OD_{562} got lower and lower. In contrast, BSA exhibited no iron binding activity. The iron chelating assay confirmed that the rMnFer2 had an iron-binding capacity.

3.6. H_2O_2 tolerance assay of *E. coli* expressing MnFer2

As shown in Fig. 4C, *E. coli* expressing MnFer2 and strain transformed with empty pET30a plasmid showed almost the same ability to survive in LB medium without or with pretreatment of 25 mM H_2O_2. When pretreated with 50 mM H_2O_2 for 1 h at 28 °C, the clone number of overnight cultured *E. coli* expressing MnFer2 was significantly more than that of the empty vector control. The results suggested that the rMnFer2 endowed *E. coli* resistance against oxidative stress induced by H_2O_2.

4. Discussion

As a ubiquitous iron storage protein, the ferritin can sequester iron in a non-toxic and biologically available form in its spherical cage like oligomer and maintain redox balance [5,37]. In vertebrates and insects, ferritins have been well investigated and known to play significant roles in detoxification, resistance against oxidative stress, as well as immune reactions [4]. However, knowledge on the function and molecular evolution of the ferritin family in crustacean keeps limited. In the present paper, we screened out and characterized all the potential ferritin subunits from the *Macrobrachium nipponense* transcriptome. Comprehensive comparison of their molecular property provides us with new insights into the evolutionary origins and functions of this pivotal protein family.

Two kinds of functionally and genetically distinct ferritin subunits, HCHs and LCHs, coexist in mammals and insects. In human, the molecular masses of H-ferritin and L-ferritin are 21 and 19 kDa respectively, and they have approximately 55% similarity in their sequences [38]. The H-subunit is primarily responsible for the ferroxidase activity of the ferritin complex, whereas the L-subunit facilitates the storage of iron into the ferritin core [5,6]. Another type subunit, the M (middle) type ferritin subunit, is reported in some vertebrates such as fish and possesses both the ferroxidase di-iron center and the ferrihydrite nucleation center [7,8]. In the present research, all the five MnFers (MnFer1-5) are predicted by the Web CD-Search Tool on NCBI to possess the speculated ferroxidase di-iron center as well the ferrihydrite nucleation center. The seven characteristic residues (E28, Y35, E62, E63, H66, E108, and Q142 by human H-type ferritin numbering) in the ferroxidase centers in vertebrate H-type ferritins are strictly conserved in the prawn ferritin subunits of MnFer1, 2 and 4. The iron binding capacity of MnFer2 as an example was confirmed through examination in vitro using purified recombinant protein rMnFer2, suggesting that the ferritin could sequester the excess iron or help absorb iron in *M. nipponense* to maintain the free iron concentration at a safe level. In contrast, MnFer3 is predicted to have a nonclassical ferroxidase diiron center with seven highlighted residues (Q24, F31, V58, A59, H62, Q104, H138). The seven conserved residues in the ferroxidase center of human ferritin H chain can be seen in most insect HCHs, but all insect LCHs retain not more than three out of seven residues [9]. We can designate MnFer1, 2, and 4 as HCH or MCH and MnFer3 as LCH with regard to the seven ferroxidase residues. Although all the four subunits of MnFer1-4 are predicted to possess a putative ferroxidase center, the proposed four characterized residues (K54, D57, E58, E61; E54, D57, E58, G61; K54, T57, V58, D61; E82, S85, E86, S89 for MnFer1-4 respectively) are quite different from those of human L-type ferritins (E57, E60, E61, and E64). The substitutions of acid Glutamate (E) by nonacid residues (K, G, T, V, S) may influence the rate of nucleation since negatively charged residues on the protein shell inner surface of L subunits are important to promote ferrihydrite nucleation. Different from ferritins of mammals and insects, most crustacean ferritins seem to be the hybrid type of H and L types [14]. However, the ferroxidase and ferrihydrite nucleation activities of crustacean ferritins require further confirmation by more experiments.

In contrast to the four subunits of MnFer1-4, MnFer5 contains a predicted Ferritin-like domain, but lacks iron ion channels, ferroxidase

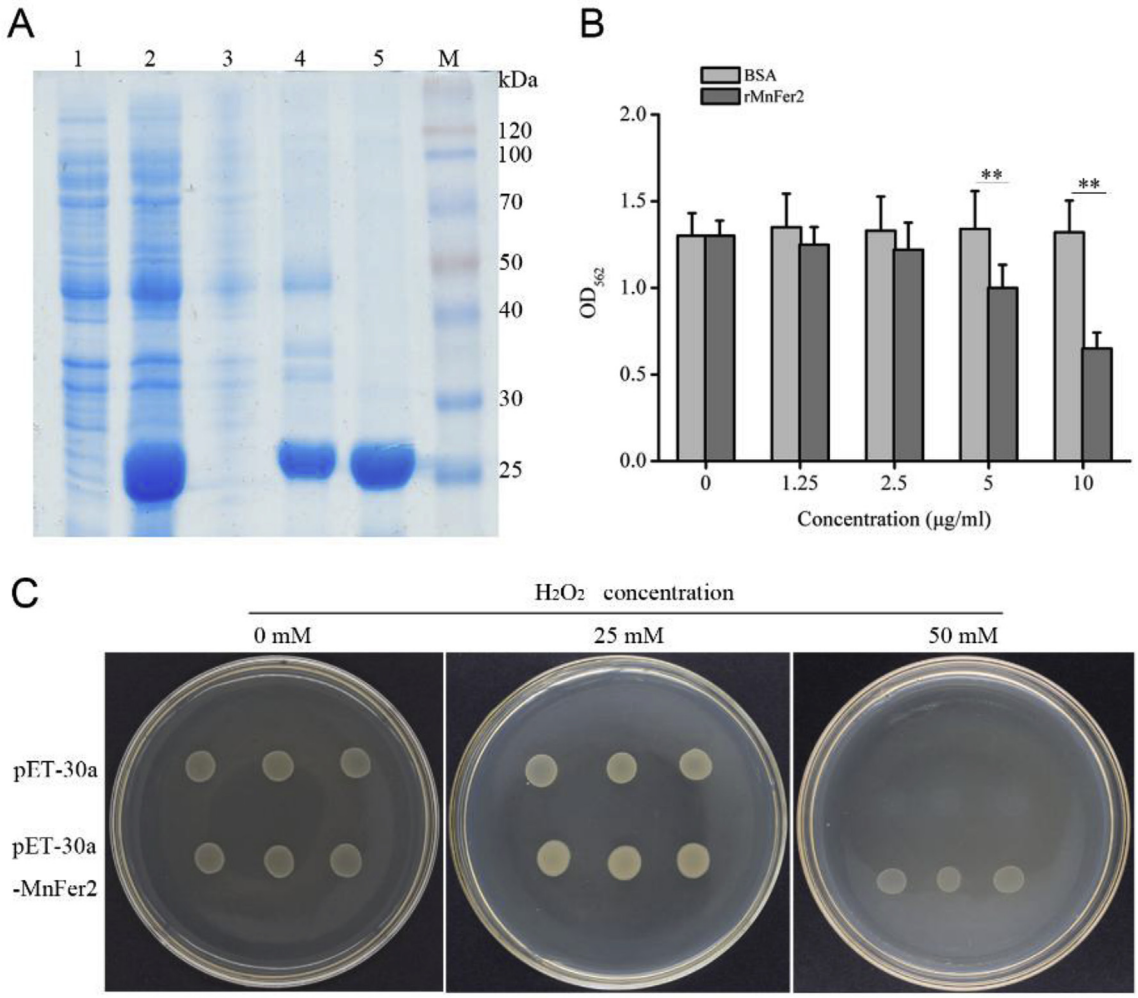

Fig. 4. (A) SDS-PAGE analysis of rMnFer2 protein. Lane 1: negative control for rMnFer2 (without induction). Lane 2: IPTG induced rMnFer2. Lane 3: supernatant of rMnFer2. Lane 4: inclusion-body rMnFer2. Lane 5: purified rMnFer2. Lane M: protein molecular standard. (B) Iron binding assay of MnFer2. Detection of Iron binding activity at different concentration of rMnFer2. The X-axis represents the concentration of rMnFer2 and BSA. The Y-axis indicates the absorbance at 562 nm after the iron-chelating reactions. (C) Tolerance of recombination strain to oxidative stress H_2O_2 on IPTG medium. The bottom row was pET-30a-MnFer2 recombinant on medium containing 0–50 mM H_2O_2, the top row on medium was the negative control strain with pET-30a.

sites, or nucleation sites. However, a distinct dinuclear metal binding motif is unexpectedly found in MnFer5, suggesting a potential correlation with metal ions. Similarly, four ferritin subunit genes have been screened out from the genome of Pacific oyster *Crassostrea gigas*. Cgi-Fer1 and Cgi-Fer2 are speculated to be M type subunits, and Cgi-Fer3 is likely to be a typical H type subunit. CgiFer4 seems to lack both a functional ferroxidase di-iron center and a ferrihydrite nucleation center, which may indicate that Cgi-Fer4 is not a typical ferritin subunit [39].

As previous studies shown, there is a vital difference between mammalian and insect ferritins. That is, mammalian ferritins serving as iron storage proteins are predominantly cytoplasmic (non-secretory), while insect ferritins are secretory and play a role in iron transport [10,40]. In contrast to insects, most crustacean ferritins are supposed to be cytosolic proteins due to lack of signal peptides [41], although a few secreted-type ferritins have also been reported in crustaceans [22]. In gastropod *Biomphalaria glabrata* [42] and mollusk *C. gigas* [39], both secretory and non-secretory ferritin subunits have been reported coexisted in one species. Here, two ferritin subunits, MnFer4 and MnFer5, contain signal peptides, suggesting that they may be secreted proteins. Prediction of sub-cellular location via CELLO and TargetP programs indicated that MnFer1 and MnFer2 are located in cytoplasm, and MnFer3 is in cell nucleus. Quite a few studies have reported the nuclear localization of ferritins, especially H-subunit, in developing neurons, hepatocytes, corneal epithelial cells, and some cancer cells. It suggests that the ferritins play a role in the regulation of iron accessibility to nuclear components, DNA protection from iron-induced oxidative damage, and transcriptional regulation [1]. Nevertheless, the nuclear localization of MnFer3 and its role in the prawn needs further experimental confirmation.

A phylogenic tree was constructed using Mnfer1-5 and 37 representative sequences of ferritins from vertebrates, mollusks, crustaceans and insects. The results revealed that secretory and non-secretory subunits were divided into two major clades. Two secretory ferritins of *Macrobrachium nipponense*, MnFer4 and MnFer5, and those of insects and other crustaceans cluster together according to their origins. The phylogenetic analysis indicated that secretory subunits have earlier origins than non-secretory ones. The non-secretory ferritin subunits of vertebrates, mollusks, and crustaceans formed three separate sub-clades within the secretory clade.

Analysis of the 5′-UTR from *M. nipponense* ferritins mRNA revealed that conserved stem loop structures (IREs) appeared in MnFer2, 4 and 5, suggesting that their expressions at translational level are controlled by iron regulatory proteins (IRP). An IRE motif was also identified in MnFer1 at the proximal site of the 5' –UTR [23]. In vertebrates, when cellular iron levels are low, the active IRP can bind the stem loop

structure (IRE) messages for both H and L chains and prevent their translation [43]. Inhibition of ferritin mRNA translation serves to control iron storage and iron transport and thereby cellular free iron levels. Although none IRE motif was found in the MnFer3 mRNA, the short 5′-UTR is not sufficient to support this claim. The ferritin mRNA of plants, yeast and bacteria, and LCH mRNA of insects lack a IRE motif [9,44]. In insects, the synthesis of HCH ferritin subunits with IRE is under both translational and transcriptional control [9,45]. IRE harbored in most of the ferritins of *M. nipponense* indicates that their synthesis is under a complex and flexible control, allowing the housefly to deal with varying iron.

Tissue-specific expression patterns of ferritins have been recorded in various animals. In the *Drosophila melanogaster*, both HCH and LCH mRNAs are much more abundant in the gut where ferritins are the key to dietary iron absorption [9,46]. In Pacific white shrimp (*Litopenaeus vannamei*), mRNA is most abundant in haemocytes [15]. In the freshwater giant prawn (*Macrobrachium rosenbergii*) and the mud crab (*Scylla paramamosain*), the ferritin predominantly transcripts in hepatopancreas, but no ferritin mRNA was detected in haemocytes [17,47]. MnFer1 mRNA is ubiquitously expressed in various tissues, although it is mainly expressed in hepatopancreas and limited in haemocytes [23]. Transcription of ferritin from the red claw crayfish (*Cherax quadricarinatus*) is widely detected in various tissues with higher expression in haemocytes, hepatopancreas and hearts followed by less expression in gills, intestines and nerves. In the present study, we detected and compared the tissue distribution of different ferritin subunits in *M. nipponense*. Diverse tissue-specific transcriptions of different ferritin subunit genes suggest that they might serve as different roles in prawns.

Other than the role in iron balance, mammalian and insect ferritins are considered an important inhibitor of free radical production, and play a role as a cytotoxic protector against oxidative stress [10]. In *Drosophila*, overexpression of both the HCH and LCH genes is required to confer protection against oxidative challenge [48]. The antioxidant properties of Crustacean Ferritin have not been demonstrated. To investigate the role of MnFers in oxidative stress, prawns were injected with Doxorubicin (DOX), an oxidative stress inducer in cells, by generating ROS and H_2O_2. The transcription of the four Mnfer genes is strongly induced by DOX at 12 h post injection, indicating the involvement of these ferritin subunits in protection from oxidative stress caused by non-iron overload. The antioxidant role of MnFer2 was further confirmed by protective test of MnFer2 against H_2O_2 in *E. coli*. The result showed that *E. coli* transformed with MnFer2 gains more resistance than that transformed with empty plasmid as control, illustrating that MnFer2 has the biological function to protect *E. coli* against oxidative stress by H_2O_2. We speculated that overexpressed MnFer2 can relieve the formation of more harmful hydroxyl radicals produced by the iron-dependent Fenton reaction. However, we didn't observe apparent iron deficiency phenotype in the MnFer2 expressing strain. It is perhaps because the recombinant proteins existed in the form of inclusion body.

Understanding of the function of ferritins has traditionally focused on their roles in iron homeostasis and redox biology. However, increasing evidence suggests that these hyperferritinemias might be involved in host defense against infection. Even though excess iron in the live cell is harmful due to its highly reactive nature, iron is an essential element for all organisms, including pathogenic microorganism. Pathogenic organisms, such as bacteria and fungi, also require iron in order to proliferate in hosts. Recently, the ferritin has been identified as an important component of innate immunity system in arthropods owing to its ability to take away available iron from invading pathogens [14]. Withholding of iron via ferritins is proposed to be one of the major strategies in innate immunity [49]. In some insects, such as *Bombus ignitus* and *Tribolium castaneum*, ferritin genes can be induced for up-regulation in response to wounding, bacterial infection or lipopolysaccharide (LPS) injection [50,51]. But in the malaria mosquito, the protein levels of LCH and HCH were decreased following bacterial injections [52]. Knockdown of ferritin genes by RNAi in the hard tick *Haemaphysalis longicornis* can result in impaired cellular immune response to injected bacteria [53]. A few of recent researches on Crustaceans also declared that ferritins could be taken as a vital immune effecter in host immune system against bacteria and virus [16,21–24,40]. We examined the expression profiles of all the four cloned ferritin genes of *M. nipponense* following bacterial challenge. The results showed that only MnFer5 was up-regulated and other subunits including MnFer2-4 were down-regulated during the early stage upon *A. hydrophila* infection. However, all the transcription levels of the four subunits restored or exceeded the unacted level at the endpoint of the experiment. In view of the fact that the ferritin expression is regulated at both transcription and translation levels, the further protein expression detection may be required for comprehensive understanding of the roles of these ferritin subunits in prawn immunity.

In summary, we screened out four new ferritin genes that encode both non-secretory and secretory subunits from the transcriptome of *M. nipponense*. Among them, MnFer2 and 4 possess a strictly conserved ferroxidase site, and MnFer3 has a non-typical ferroxidase site. In contrast, MnFer5 is a nonclassical number of the ferritin family, which has a distinct dinuclear metal binding motif, but lacks an iron ion channel, a ferroxidase site, or a nucleation site. The wide distribution in various tissues and strong inducibility by bacterial infection make MnFer5 even more mysterious. Systematic analysis of these molecular characteristics and potential functions in prawns shows that crustacean ferritins have some unique features different from their mammalian and insect counterparts. Our results suggest a protective role of ferritins from *M. nipponense* in iron homeostasis, redox biology and antibacterial immunity.

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