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A novel ferritin gene from *Procambarus clarkii* involved in the immune defense against *Aeromonas hydrophila* infection and inhibits WSSV replication

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ABSTRACT

Ferritin is a protein related to the storage of iron and widely distributed in animals. It participates in many biological process, including antioxidation, cell activation, angiogenesis, regulation of iron metabolic balance and immune defense. In the present study, a novel ferritin gene was identified from red swamp crayfish *Procambarus clarkii*, with a cDNA sequence encoding a predicted 221 amino-acid residues. The ferritin protein contains a 19-residue signal peptide and 145-residue classic ferritin domain. The NJ phylogenetic analysis showed *Pc*Fer clustered with other crustacean peptides. The recombinant *Pc*Fer protein was produced and purified in *E. coli*, and the anti-rabbit polyclonal antibody was obtained. The rPcFer exhibited iron binding activity at a dose-dependent effect. The qPCR and western blot analysis revealed that *PcFer* was highly expressed in hemocytes, hepatopancreas, and gills. After challenged with WSSV and *Aeromonas hydrophila*, the mRNA and protein expression patterns of PcFer were significantly up-regulated in hemocytes and hepatopancreas. dsRNA interfering technique was utilized to silence the expression of PcFer gene. The WSSV copy number in PcFer silenced shrimp was much higher than that in the control group. The present study indicated that PcFer was involved in the immune defense against WSSV and *Aeromonas hydrophila*, and might inhibit WSSV replication in *P. clarkii*. These results will provide important data support for further study of the functional role of the ferritin gene.

1. Introduction

Iron is involved in many life activities among living organisms, including oxygen transport, DNA replication, photosynthesis, metabolic redox process and cell proliferation. It plays an important role in maintaining normal cell growth and metabolism [1]. However, excessive iron content in the cells can cause certain damage and death to the body cells [2,3]. Maintaining the metabolism and homeostasis of iron in living cells has important significance. Ferritin is a protein related to the storage of iron and widely distributed in animals. Recent studies have shown that ferritin participates in many biological process, including antioxidation, cell activation, angiogenesis, elimination of the toxicity of some heavy metals and regulation of iron metabolic balance [4–6]. Ferritin could store the iron by binding excess free Fe(II) ions in cellular environments and releasing them only in times of iron shortage [7]. So ferritin protein plays vital roles in maintaining the normal growth of living organisms.

Ferritins have been widely distributed in bacteria, fungi, plants, invertebrates and vertebrates [8]. It has been identified and characterized from some crustaceans, such as *Litopenaeus vannamei* [9,10], *Fenneropenaeus chinensis* [11], *Penaeus monodon* [12], *Eriocheir Sinensis* [13], *Macrobrachium rosenbergii* [14], *Macrobrachium nipponense* [15]. The mRNA expression level of these ferritins could be significantly regulated under pH stress, high heavy metal concentration, and thermal stress. A lot of studies revealed ferritin participated in the iron storage and anti-oxidative stress [16], but the recent researches are paying more and more attention to the role of ferritin in the host innate immune response. Ferritin was involved in the immune response as an acute phase reaction protein during the pathogens invading [17]. It could exhibit resistant activity to bacterial growth [13,18]. In addition, ferritins were also involved in the innate defense against virus. Some viral pathogens can promote the proliferation and diffusion by

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Table 1
Details of the primer pairs used in this study.

Primers	Sequences (5′-3′)	Efficiency
18S-F	TATACGCTAGTGGAGCTGGAA	96.4%
18S-R	GGGGAGGTAGTGACGAAAAAT	
PcFer-F	ACTCAGTGTAGTGCCGTAG	
PcFer-R	ATTGCTAACATATTGTAAA	
qPcFer-F	CTCAGTGTAGTGCCGTAG	96.8%
qPcFer-R	ATGAAGCAGGATGAAGAT	
ExpPcFer-F	GCCgaattcAGATCTAGAATACAACTGC	
ExpPcFer-R	CGGctcgagTGGTACAATGTATAAGATT	
dsPcFer-F	TAATACGACTCACTATAGGGTCCCCTTGATTACCGACCC	
dsPcFer-R	TAATACGACTCACTATAGGGGGCTGGTTGAAAGGTTGAT	
dsEGFP-F	TAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC	
dsEGFP-R	TAATACGACTCACTATAGGGAGTTCACCTTGATGCCGTTCTT	
VP28-F	AAACCTCCGCATTCCTGTGA	
VP28-R	TCCGCATCTTCTTCCTTCAT	
M13-F	CGCCAGGGTTTTCCCAGTCACGAC	
M13-R	CACACAGGAAACAGCTATGAC	
T7	TAATACGACTCACTATAGGG	
T7 Ter	TGCTAGTTATTGCTCAGCGG	

disturbing the iron homeostasis of host cell [19,20]. The gene expression level of ferritin could be up-regulated in yellow head virus (YHV) infected *P. monodon* [21], and acute viral necrobiotic virus challenged *Chlamys farreri* [22]. These studies indicated ferritin's participation in the response to viral infection, but the specific mechanism was still not clear.

The red swamp crayfish *Procambarus clarkii*, widely distributed in the natural environment, has become an important economic breeding species in China. However, with the pollution of water environment, the increase of the breeding density, and the degradation of germplasm, many bacterial and viral diseases have brought huge economic losses to crayfish farming industry. WSSV is traditionally considered as a highly pathogenic and prevalent virus to the breeding penaeid prawns [23]. Although it can be carried by most crustaceans, it only produces lethal effects to certain species [24]. The WSSV could infect the most organs and cause high mortality in *P. clarkii* [25]. So *P. clarkii* are considered as an alternative experimental host in the study for WSSV [26]. In the actual breeding, the disease caused by WSSV to crayfish is becoming more and more serious. To effectively control the outbreak of disease, it is necessary to study the immune system of crayfish. In the present study, we study the function of ferritin, analyze its expression profiles under different pathogens challenge, obtain its recombinant protein and polyclonal antibodies, and silence its expression by dsRNA interference. These results will provide important data support for further study of the functional role of the ferritin gene in *P. clarkii*.

2. Materials and methods

2.1. Animals, challenge experiments, and sample collections

Red swamp crayfish with average body weight of 7.28 ± 1.62 g were obtained from a crayfish farm in Yangling, Shaanxi Province, China. The crayfish were reared in water tanks at the temperature of 23 °C and pH 7.2. They were fed with commercial pellets twice per day and maintain optimal dissolved oxygen in the laboratory under optimal rearing conditions. The crayfish were randomly selected for WSSV detection as previous study [27]. Nine individuals were randomly taken for tissue preparation (including gill, nerve, muscle, stomach, hepatopancreas, heart, and intestines). Hemolymph was collected from the ventral sinus located at the first abdominal segment using a syringe with equal volume of anticoagulant-modified Alsever solution [28]. Hemocytes were isolated by centrifugation at 800 g, 4 °C for 10 min. The different tissues were dissected out and preserved in liquid nitrogen for RNA and protein extraction.

Healthy juvenile shrimp were used for WSSV and *A. hydrophila* challenge. For WSSV challenge, 100 μL 1×10^5 copies WSSV particles were intraperitoneal injected into each crayfish. The WSSV particles were prepared according to previous studies [29]. For *A. hydrophila* challenge, 100 μL 1.0×10^7 cfu/mL bacterial solutions were intraperitoneal injected into each crayfish. The bacteria strain was isolated from *P. clarkii* and saved by our lab. As the negative control, the same volume of sterile PBS was used to inject into the crayfish. After 3, 6, 9, 12, 24 h post-injection, three different hemocytes and hepatopancreas samples were prepared. Each sample was collected from three individuals. All the dissected samples were flash-frozen in liquid nitrogen and stored at −80 °C.

2.2. Preparation of total RNA, cDNA synthesis, and cloning of PcFer gene

Total RNA was extracted from the different samples using Trizol reagent (Takara, Japan) following the manufacturer's protocol. The RNAs were quantified using a NanoDrop 2000 spectrophotometer. Before cDNA synthesized, the genomic DNAs were removed from RNA by DNase (Takara, Japan). cDNA samples were synthesized by PrimeScript RT Enzyme following the manufacturer's protocol (Takara, Japan).

One pair of specific primers were designed to amplify the predicted cDNA sequence (shown in Table 1). PCR amplification was performed using the cDNA from hepatopancreas as template. The PCR program was performed as following: 1 cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, followed by the final extension at 72 °C for 10 min. The PCR products were sub-cloned into pMD20-T vector (Takara, Japan) and transformed into TOP10 *Escherichia coli* (Tiangen, China) for sequencing.

2.3. Sequence analysis

The ORF of *Pc*Fer was identified by ORF Finder at the National Center for Biotechnology Information. The cDNA and deduced amino acid sequence of *Pc*Fer were analyzed using DNAstar and BLAST. Signal peptide was analyzed using the program SignalP (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight (MW) of the protein was calculated based upon its constituent amino acids, using the compute pI/MW software tool. Phylogenic tree was constructed on the basis of the deduced amino acid sequences by the neighbor-joining (NJ) within MEGA version 5.0 with 1000 replicates. The function domains were presumed using SMART prediction (http://smart.embl-heidelberg.de/) [30].

2.4. Tissue distribution and post-stimulation expression profiles of PcFer mRNA

Relative quantitative real-time PCR assay (qPCR) was performed to analyze the expression patterns of *Pc*Fer in different tissues, and hepatopancreas and hemocyte injected with WSSV and *A. hydrophila*. 18S RNA was used as the reference gene. The qPCR of specific primer pairs were listed on Table 1. The efficiencies of each pair of primers were analyzed following the method [31]. The predicted sizes of the PCR products were firstly sequenced to confirm the specificity and effectiveness of primers for real-time PCR. The real-time PCR was performed on a CFX96 (Bio-Rad) using the SYBR qPCR Mix with reaction conditions as recommended by the kit manufacturer. The programs for qPCR were carried out as follows: 94 °C for 5 min, 40 cycles of 94 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s. A melting curves analysis was performed over a ranged of 60–90 °C. The relative expression level of target gene was calculated using the comparative Ct method with formula $2^{-\triangle\triangle Ct}$. The results were expressed as mean ± S.D. The different reactions for qPCR detection were repeated in triplicate.

After WSSV and *A. hydrophila* challenge, the hemocytes and hepatopancreas samples were also selected to analyze the expression of *Pc*Fer at 3, 6, 9, 12, 24 h post-injection. The qPCR operations were performed as describe above. The relative expression level were expressed as mean ± S.D. Data were analyzed with one-way analysis of variance (ANOVA) and Duncan's Multiple Comparisons.

2.5. Recombinant expression and purification of PcFer protein

In order to obtain the recombinant *Pc*Fer protein, the coding sequence of the *PcFer* gene was amplified using the *ExpFer-F* and *ExpFer-R* primers (shown in Table 1). The *Eco*R I and *Xho I* restriction enzyme sites were shown underline. The strategy for the construction of the expression plasmid was performed according to previous study [32]. After sequenced by company, the recombinant vector pET32a/*Pc*Fer was transformed into *E. coli* BL21 (DE3) plysS competent cells. Expression of the fusion protein (His-*Pc*Fer) was induced by 1 mM IPTG at 37 °C for 4 h. Then bacteria cells were harvested and disrupted by sonication in ice bath. The His-tag rPcFer protein was purified using Ni^{2+} affinity column according to previous study [33]. The expression analysis of the recombinant protein was analyzed by SDS-PAGE electrophoresis. Concentration of the rPcFer was tested by Bradford Assay Kit.

2.6. Preparation of antibody and western blot analysis

The purified r*Pc*Fer protein was used for preparing the polyclonal antibodies in rabbits in Genscript Company (Nanjing, China). The recombinant protein in T-MaxTM adjuvant was used to immunize rabbits subcutaneously. The IgG fraction was purified using antigen affinity chromatography. Enzyme-linked immunosorbent assay (ELISA) was used to determine the titer of antibody. The specificity of antiserum was tested by western blotting.

The protein expression profiles of *Pc*Fer among different tissues (muscle, gill, hemocyte, stomach, hepatopancreas and intestine) were detected by western blot analysis. Total protein was extract from different tissues of crayfish without any treatment by Total Protein Extraction Kit (BestBio, China) following the manufacturer's protocol. The protein concentrations were normalized to 5 μg/μl using the Bradford Assay Kit. After SDS-PAGE electrophoresis, different protein samples were transferred onto PVDF membrane (Millipore, USA). Then the membrane was blocked by 5% nonfat-milk for 2 h at room temperature. After incubation by rabbit anti-PcFer antibody or β-tubulin antibody, the membranes were washed by TBST for three times. Then the anti-rabbit IgG-HRP labeled was used as the second antibody. After three washes, the immunoreactive bands were visualized using the enhanced chemiluminescence reagents (Advansta, California, USA) according to manufacturer protocol, and quantified by chemiluminescence imaging system (Bio-Rad, California, USA).

2.7. Preparation of double strand RNA (dsRNA) and RNA interference

The RNA interference assay was performed to knockdown the *Pc*Fer gene by the double-strand RNA (dsRNA). Two pairs of primers with T7 promoter sequences, dsPcFer-F/dsPcFer-R were designed to amplify two cDNA fragments of PcFer gene. The primers with T7 promoter sequences were shown on Table 1. The PCR amplification program was as follows: denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s; final extension at 72 °C for 10 min. The purified PCR products were used to synthesize the corresponding dsRNAs using TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific, USA). DsRNA of EGFP was synthesized based on the template of pEGFP-N1 plasmid. The synthesized dsRNA was treated with ribonuclease (RNase) A (TaKaRa, China) to digest the single strand RNA and examined by 1.2% agarose gel electrophoresis. The concentrations of synthesized dsRNAs were assessed by Nanodrop 2000. All the dsRNAs were stored at −80 °C for further use. 4 μg dsRNA was injected into the abdominal segment of each shrimp. At 48 h and 72 h after dsRNA injection, hepatopancreas of crayfish in different groups were isolated and used for detection of the *Pc*Fer expressions. The protein expression levels of PcFer were also detected using anti-PcFer polyclonal antibody.

2.8. Detection of the WSSV copy number in ferritin silenced shrimp

After dsRNA interference in crayfish for 48 h, the crayfish were injected into 100 μL (1◊10^5 copies) WSSV solution. Two groups were divided including control groups (dsEGFP) and experimental group (dsPcFer). And the time point of WSSV injection was recorded as 0 h. The muscle of 9 individuals from each group were collected at 0, 24 and 48 h after WSSV injection. The different samples were used for DNA extraction to detect the WSSV copy numbers in each shrimp. The DNA was extracted using Genomic DNA Kit (Tiangen, China). The WSSV copy number was quantified by quantitative real-time PCR with VP28-F and VP28-R primers. The concentrations and copy numbers of standard samples pMD19T-VP28 were calculated. Then four-fold serially diluted solutions of plasmid were used as standard samples to generate a standard curve in the quantitative real-time PCR, performed with specific VP28 primers to measure the viral copy number. Different samples of WSSV copy number were calculated as per ng pleopods DNA based on the standard curve.

2.9. Iron binding activity of the recombinant PcFer protein

The iron binding activity of the purified rPcFer was determined according to previous study [34–36]. The purified rPcFer, rTrx protein (produced by empty pET32a vector), and heat-denatured rPcFer were adjusted the concentrations of 2, 4, 6, 8, 10 μg/ml. Briefly, 1 ml of each solutions were added to a solution of 20 μl of 2 mM $FeCl_2$. The reaction was initiated by adding 40 μl of 5 mM ferrozine and incubated for 10 min at room temperature. Then the absorbance was measured at 562 nm. Three replicates were performed for each assay.

2.10. Statistical analysis

During the experiment, all the assays described above were biologically repeated for three times. The numerical experiment data were present as mean ± standard deviation (SD). The statistical analyses were carried out with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The statistical significance was analyzed with ANOVA defined as $p < 0.05$.

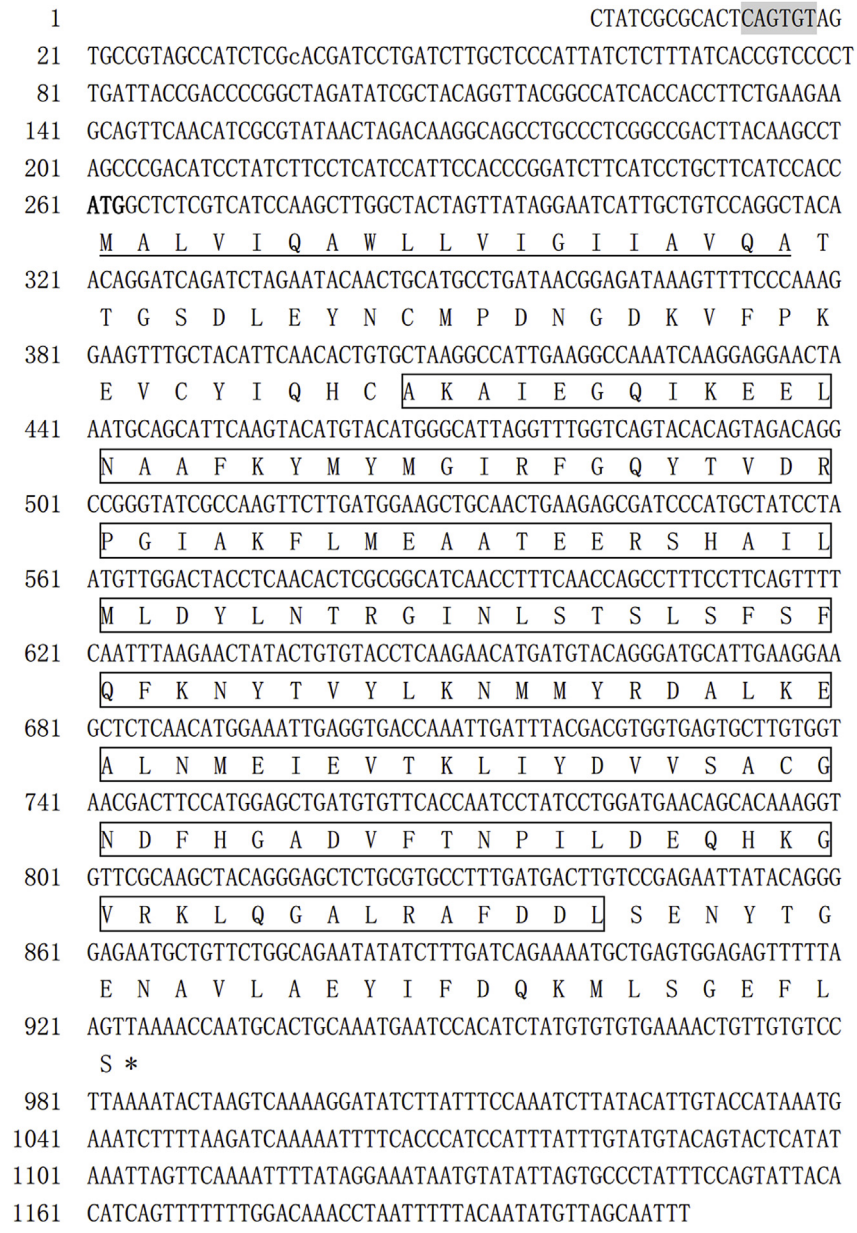

Fig. 1. The cDNA and deduced amino acid sequences of *Pc*Fer. The start codons are in bold. The stop codons are indicated by asterisk. The signal peptide sequences are underlined. The putative ferritin domain is indicated in box. The iron responsive element in the 5′-UTR is in grey.

3. Results

3.1. Identification and sequence analysis of PcFer

The cDNA sequence of the ferritin gene was 1208 bp, with an ORF 663 bp, 5′-UTR 261 bp and 3′-UTR 282 bp. The specific primers shown in Table 1 was used to amplify the *Pc*Fer gene. The deduced amino acid sequence contains 221 residues. The predicted molecular weight was about 25.02 kDa and theoretical pI was 4.95. The cDNA and deduced amino acid sequences of *Pc*Fer were shown in Fig. 1 A signal peptide with 19 amino acid residues was located in the N-terminal of ferritin protein. Prosite motifs analysis by SMART program showed that a typical ferritin domain was found from 49 to 194 residues. A putative iron responsive element (IRE) was observed in the 5′-UTR with a well-conserved 5′-CAGUGU-3′ sequence [1]. No transmembrane region was found under TMHMM analysis. The sequence has been submitted to NCBI GenBank with the accession number MH992753. The phylogenetic relationship tree of PcFerritin to those from other species was constructed using the neighbor-joining method. As shown in Fig. 2, *P. clarkii* ferritin shared a closer relationship with the other invertebrate ferritin from *E. sinensis, F. chinensis, M. nipponense*, and so on. The ferritin from mammals and teleosts are assigned to the distinct clusters respectively.

3.2. Preparation of recombinant proteins and polyclonal antibody

To obtain the recombinant *Pc*Ferritin protein, 1 mM IPTG was used to induce the expression of fusion protein in *E. coli* BL21. As analysis by SDS-PAGE (shown in Fig. 3), compared to the control, an apparent protein band with a molecular weight of approximately 45 kDa was identified after Coomassie blue staining. The molecular weight of the recombinantly expressed protein is consistent with the predicted size (with the tag protein of vector about 20 kDa). Then after disruption by sonication in an ice bath, the r*Pc*Fer was purified from the supernatant solutions by Ni^{2+} affinity chromatography column. As shown in Fig. 3, a precise band without other protein bands were detected indicating the

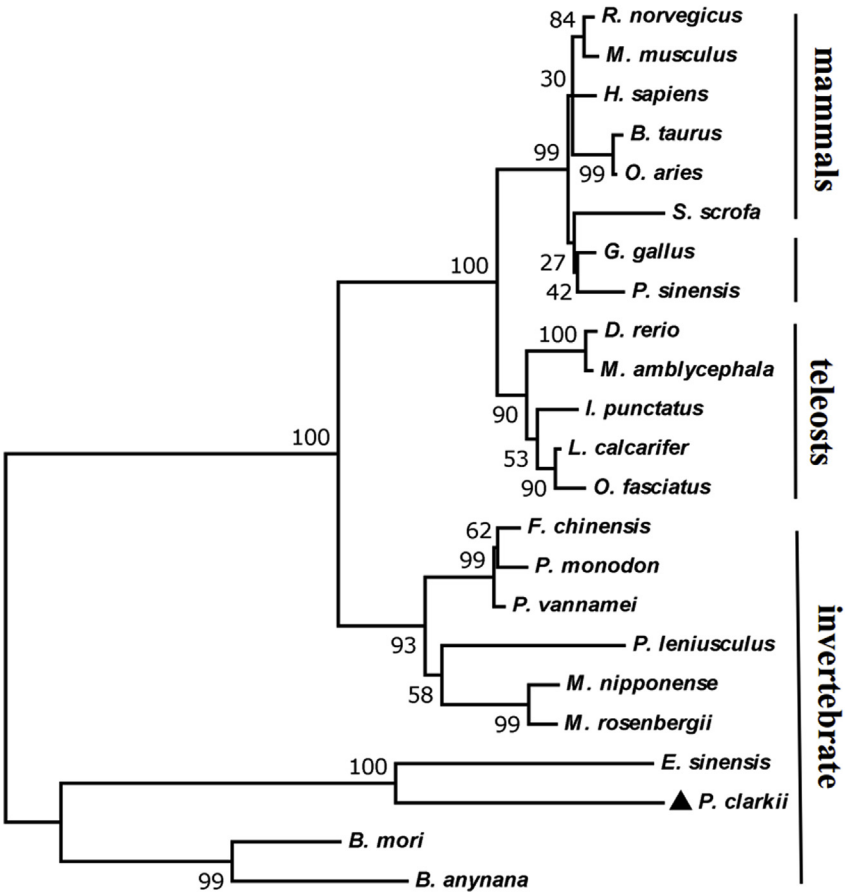

Fig. 2. The phylogenetic NJ tree from PcFerritin and other organisms. The bootstrap was set at 1000. The PcFerritin is labeled with triangle (▲). Species and gene accession numbers are as follows: *Rattus norvegicus* (AAH81845), *Mus musculus* (NP_034369), *Homo sapiens* (AAH16857), *Bos taurus* (AAI51550), *Ovis aries* (NP_001009786), *Sus scrofa* (NP_999140), *Gallus gallus* (NP_990417), *Pelodiscus sinensis* (XP_006111800), *Danio rerio* (NP_571660), *Megalobrama amblycephala* (ALG05437), *Ictalurus punctatus* (ADE09343), *Lates calcarifer* (ADU87113), *Oplegnathus fasciatus* (BAM37461), *Fenneropenaeus chinensis* (ABB05537), *Penaeus monodon* (ABP68819), *Penaeus vannamei* (AAX55641), *Pacifastacus leniusculus* (CAA62186), *Macrobrachium nipponense* (AHJ90472), *Macrobrachium rosenbergii* (ABY75225), *Eriocheir sinensis* (ADF87491), *Bombyx mori* (NP_001037580), *Bicyclus anynana* (XP_023945374).

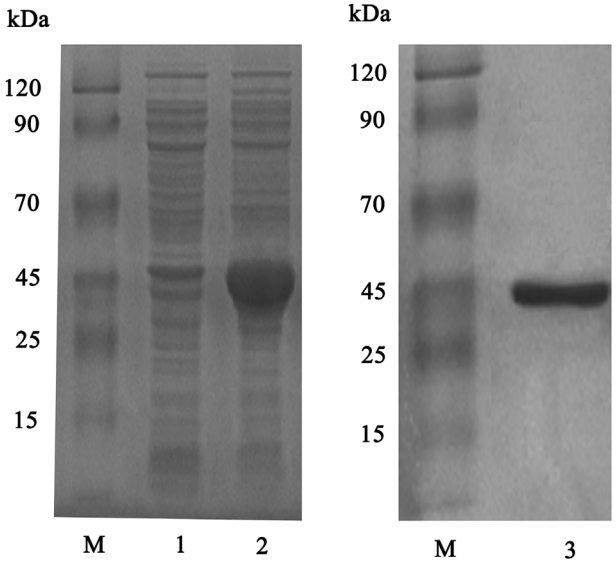

Fig. 3. Detection on the recombinantly expressed PcFerritin. Lane M represented molecular mass standards. Lane 1 showed expressed protein without IPTG induction. Lane 2 showed expressed protein after IPTG induction. Lane3 showed purified PcFerritin protein.

recombinant protein was successfully purified. The concentration of the recombinant protein was 1.6 mg/ml. After purification using antigen affinity chromatography, ELISA showed that the titer of the anti-rabbit polyclonal antibody was 1: 352,000.

3.3. Expression profiles of PcFer in different tissues and after WSSV and A. hydrophila infections

The mRNA and protein expression levels of *PcFer* among different tissues including hemocyte, nerve, muscle, stomach, hepatopancreas, heart, intestines and gill were detected by qPCR and western blot. 18S and β-tubulin were used as reference respectively. From the results (shown in Fig. 4A), *PcFer* was expressed in various tissues and presents a constitutive expression distribution. However, the mRNA expression levels are highest in hemocytes and hepatopancreas, moderate expression levels in muscle, intestine, stomach, and gill, and the lowest level in nerves and muscles. We also detected the protein expression level by western blot method. As shown in Fig. 4B and C, the ferritin protein was highest expressed in hemocyte, hepatopancreas, and gill, lowest level in stomach. Protein expression levels are basically consistent with mRNA expression levels.

The expression level changes of mRNA and protein of ferritin in hemocyte and hepatopancreas were detected after WSSV and *A. hydrophila* infections. As shown in Fig. 5A and Fig. 5B, the mRNA expression level of ferritin in hemocyte and hepatopancreas could be significantly up-regulated at short time after *A. hydrophila* infection. WSSV could also induced the mRNA expression of ferritin in hemocyte and hepatopancreas (shown in Fig. 5C and D). But this induction effect may be related to the amount of virus. The ferritin protein expression levels in hepatopancreas after infections were also analyzed. As shown in Fig. 5E and F, the amount of ferritin protein has no significantly changed during 1–6 hpi, while up-regulated at 12 and 24 hpi. After WSSV infection, ferritin protein was up-regulated at 6 and 24 hpi, while no significantly changed during 3 and 12 hpi.

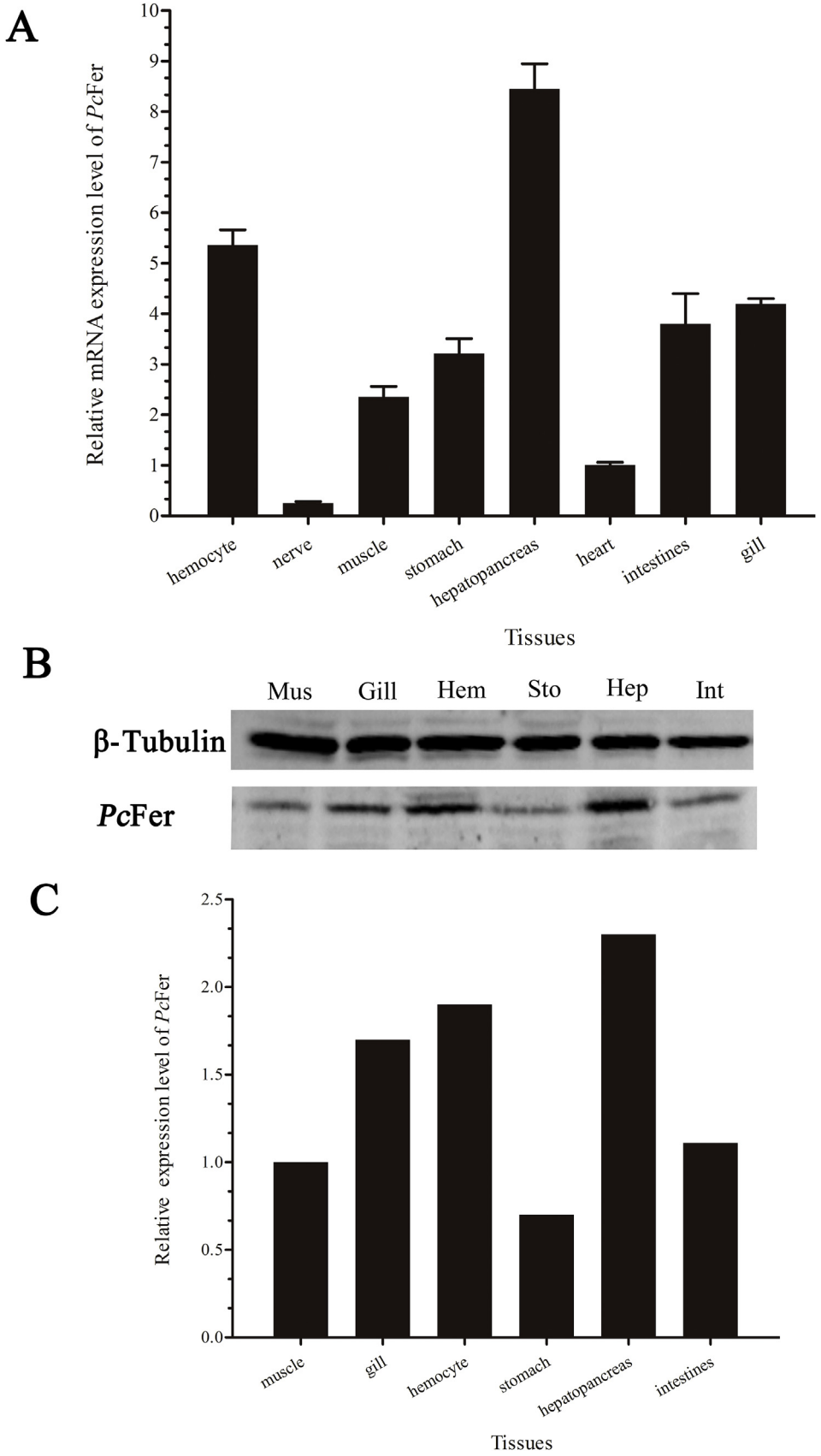

Fig. 4. Expression profiles of PcFer in different tissues. (A) mRNA relative expression level of PcFer. 18S was used as the reference gene. The expression was calculated using the $2^{-\triangle\triangle Ct}$ method. (B) Protein expression level of PcFer in different tissues by western blot analysis. Mus-muscle, Hem-hemocyte, Sto-stomach, Hep-hepatopancreas, Int-intestines. β-tubulin was used as the reference gene. (C) Quantity analysis of PcFer in (B).

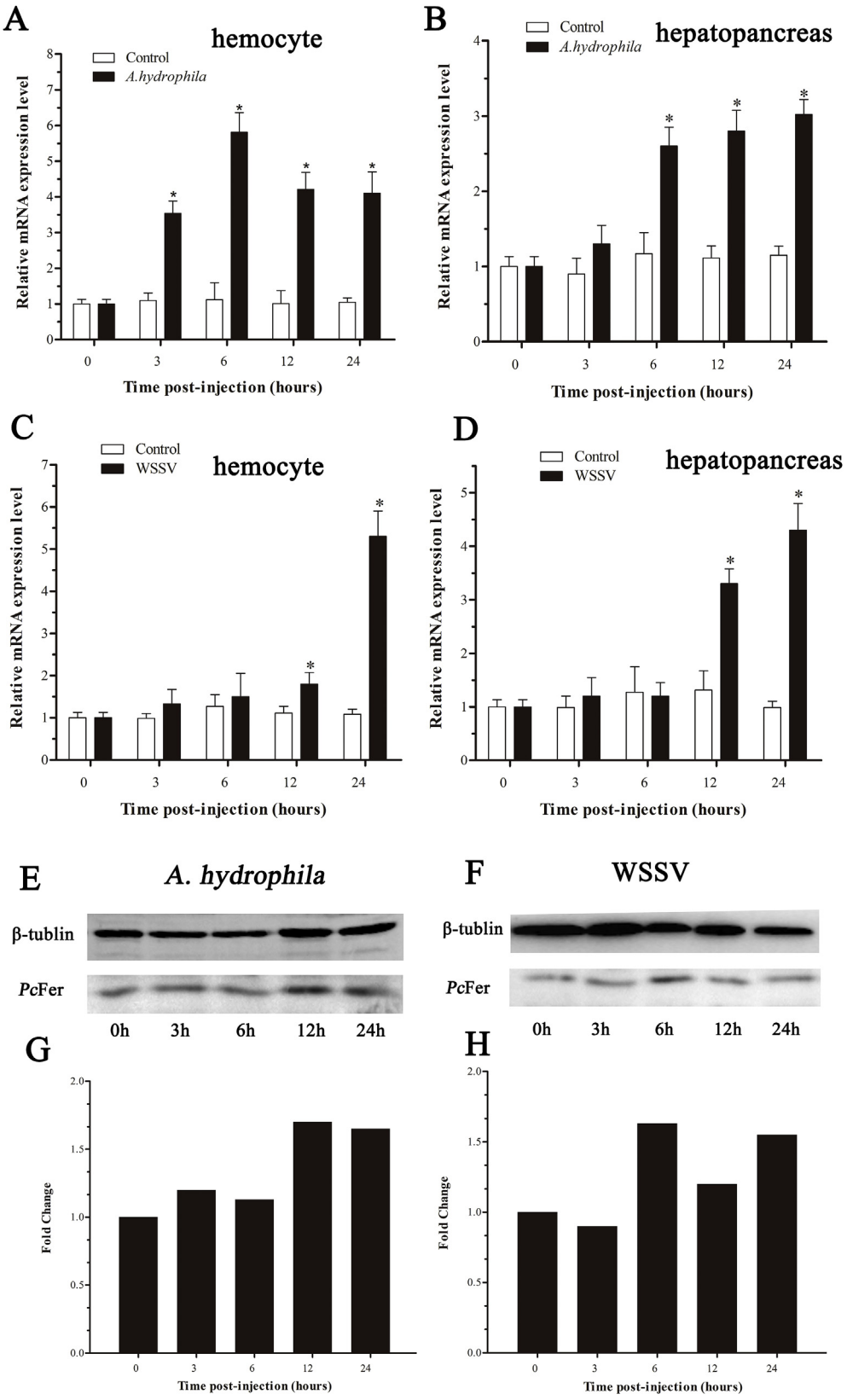

Fig. 5. Protein and mRNA expression levels of PcFer in hemocyte and hepatopancreas after WSSV and *A. hydrophila* infections. (A) and (B) qPCR analysis of *PcFer* transcripts in different time points after *A. hydrophila* infection in hemocyte and hepatopancreas, respectively. (C) and (D) qPCR analysis of *PcFer* transcripts in different time points after WSSV infection in hemocyte and hepatopancreas, respectively. (E) and (F) Western blot analysis of PcFer proteins in hepatopancreas after WSSV and *A. hydrophila* infections at different time points. (G) and (H) The band intensities quantity analysis of PcFer in (E) and (F), respectively. Bars with asterisk * indicated significantly different ($p < 0.05$).

3.4. Detection on the silencing efficiency of PcFer

The dsRNA was utilized to interference the expression of *Pc*Ferritin. The dsEGFP was used as the negative control. After injection of dsRNA at 48 and 72 h, the mRNA and protein expression level of ferritin in hepatopancreas were detected respectively. From these results (shown in Fig. 6A), the expression of *Pc*Fer was lower than controls, indicating the silence of *Pc*Fer gene. The protein expression of PcFer was also analyzed by western blot method. As shown in Fig. 6B and C, the expression level had no significantly changes after dsEGFP injection at 48

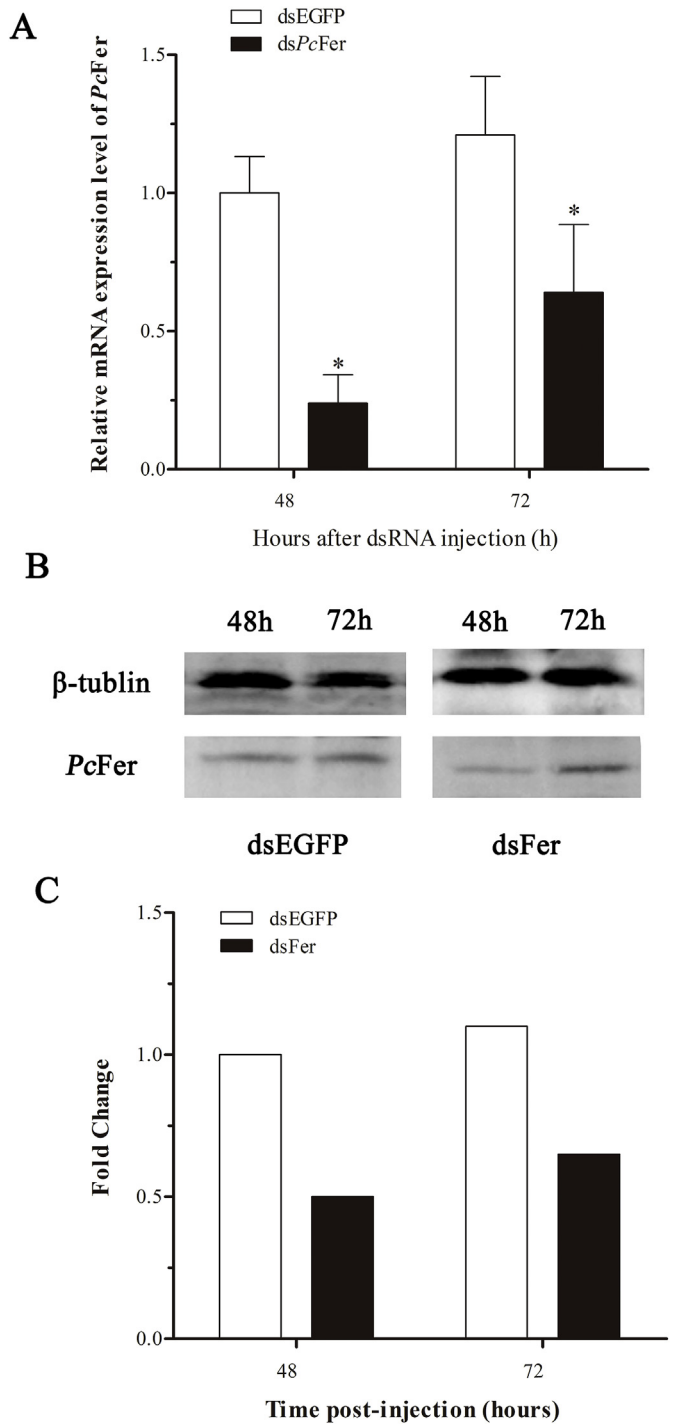

Fig. 6. Expression level of PcFer in hepatopancreas at 48 h and 72 h after injection of dsRNAs. The same dosage of EGFP dsRNA was used as negative control. (A) qPCR analysis of *PcFer* transcripts at 48 h and 72 h. (B) Protein expression levels of PcFer at 48 h and 72 h. β-tubulin was used as the reference gene. (C) The fold change of the PcFer protein expressions compared with the negative control based on the band intensities quantity analysis. Bars with asterisk * indicated significantly different ($p < 0.05$).

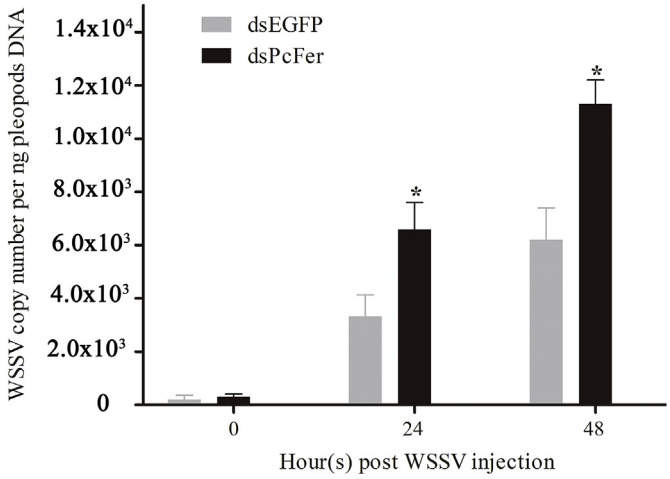

Fig. 7. Amount of WSSV particles in gene-silenced shrimp at 24 and 48 h after WSSV injection. dsEGFP group was used as the negative control. The WSSV copy numbers were detected by qPCR. Asterisk (*) indicate significant differences ($p < 0.05$).

and 72 h. But the ferritin expression level was significantly changed after dsFer injection, compared with the control.

3.5. Detection on the viral loading number in PcFer interfered shrimp during WSSV infection

To learn the function of PcFerritin during WSSV replication, the amount of WSSV in *PcFer* interfered shrimp muscle were also detected by quantitative real-time PCR. As shown in Fig. 7, the amount of WSSV has increased as time dependence in both control group and experimental group. But the WSSV copy numbers in dsPcFer group were significantly higher ($P < 0.05$) than dsEGFP groups at both 24 and 48 h post WSSV injection.

3.6. Iron binding activity of rPcFer protein

The purified rPcFer protein was used to detect the iron binding activity at different concentrations. As shown in Fig. 8, after incubation with rPcFer, the OD_{562} values decreased with the increasing concentration recombinant. The iron binding activity of rPcFer showed a

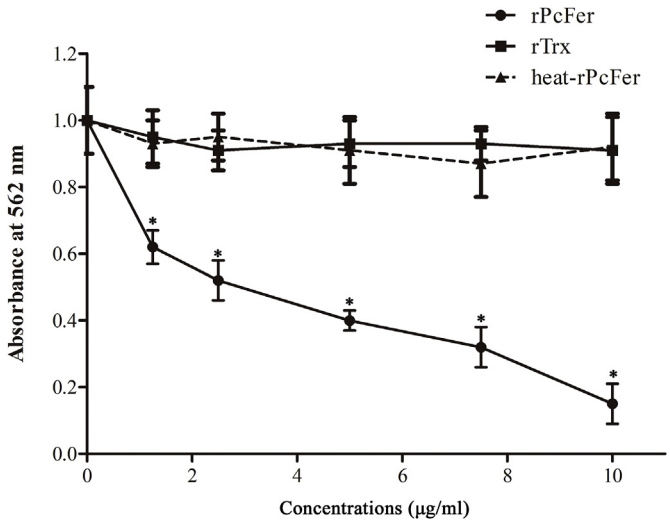

Fig. 8. Iron binding activity of rPcFer. The abscissa represents the concentrations of different rPcFer, rTrx and heat-rPcFer. The ordinate indicates the optical density at 562 nm. Each bar represented the mean ± SD (n = 3).

dose-dependent effect. The rTrx protein and heat-rPcFer protein were used as negative control. From the results, they showed no significant difference after incubation with solutions, indicating no iron binding activity.

4. Discussion

In the present study, a ferritin gene was clone from *P. clarkii*. Through the sequence structure and homologous phylogenetic analysis, the PcFer exhibited a classical ferritin structural features as the ferritin sequences of other species. During the 5′-UTR, an IRE structure was also found in PcFer. At the translational level, the synthesis was controlled through the iron regulatory proteins (IRPs) by binding to IREs [37]. IRPs senses the state of iron in the cytoplasm through the Fe-S switching mechanism [38]. The same structural features indicated its potential regulation mechanism of ferritin in *P. clarkii*. Ferritin is widely distributed among the species, but it has distinguished expression profiles among different animals. In *Sciaenops ocellatus*, ferritin was mostly abundant in liver and blood [39], while it was in muscle and spleen from *Scophthalmus maximus* [40]. In *Salmo salar* the highest expression level of ferritin was in gonad [41]. In the present study, the mRNA and protein expression level of *PcFer* was abundant in hepatopancreas and hemocyte. This was consistent with the study of ferritin in *Exopalaemon carinicauda* [36]. It is well known that the liver is the main organ for iron metabolism and synthesis of iron storage proteins. The high expression of ferritin in hepatopancreas is suggested to be related to the storage of irons. Hemolymph plays important role in the innate immunity of crustaceans and is widely involved in cellular and humoral immunity [42]. The abundant of ferritin in hemocyte indicated its potential immune defense functions in *P. clarkii*.

Till present, studies of pathogens infection that induce up-regulation of ferritin have been reported in many species, including fish, amphibians, crustaceans, and molluscs. The lipopolysaccharide could induce ferritin expression and lower free available iron levels in the presence of ample oxygen [43]. A challenge with *Vibro anguillarum* resulted in time-dependent significant up-regulation of ferritin mRNA in *Scapharca broughtonii* [18]. The expression of ferritin could be significantly up-regulated under *A. hydrophila*, iron and H_2O_2 stress in *Megalobrama amblycephala* [35]. In the present study, it was also confirmed that the expression of ferritin in crayfish hemocytes and hepatopancreas were significantly up-regulated after *A. hydrophila* and WSSV infection. This up-regulation indicated ferritin is involved in the body's immune defense process in *P. clarkii.* As previous studies, iron was an essential element for most microbial growth and maintenance of pathogenicity [44]. When the pathogen infects the host and adheres to the cell surface, the host cell may regulate the iron ion concentration through the expression of ferritin, thereby inhibiting the proliferation of the pathogen [45]. From our study, the iron binding activity of rPcFer was clearly confirmed. Some study has also demonstrated that ferritin could directly act as an immune effector to exhibit certain antibacterial activity [18]. In addition, It has also demonstrated that the antibacterial ability of ferritin is also achieved by reducing the adhesion of Gram-negative bacteria and Gram-positive bacteria to the cell surface [46]. These results confirmed that the ferritin is involved in the host antibacterial immune response, but which mechanism is the primary one needs further study.

WSSV is a highly pathogenic and prevalent virus that threatens shrimp farming. Although its lethality to crayfish was not as high as the mortality rate of prawn shrimp, WSSV has brought huge economic losses to the crayfish industry in recent years. In the study of *F. chinensis* [11], *M. japonicas* [47] and *L. vannamei* [48], it was confirmed that WSSV infection can up-regulate the expression of ferritin. In red claw crayfish *Cherax quadricarinatus*, the ferritin can inhibit the replication of WSSV in haematopoietic (Hpt) cells by regulating iron ions [49]. Some studies demonstrated that ferritin administration effectively enhances immunity, physiological responses and survival of *L. vannamei* challenged with WSSV [50]. In the present study, besides the ferritin could be up-regulated by WSSV at mRNA and protein levels, the replication of WSSV was significantly increased after interference with dsRNA. The results indicate that inhibition of ferritin expression facilitates viral replication, suggesting its potential role in inhibiting viral replication. It was consistent with those previously observed in the shrimp, which confirmed the anti-WSSV function of ferritin [51]. The host could use an iron withholding mechanism to restrict the availability of this essential nutrient to the invading virus. WSSV protein kinase 1 (PK1) could interacted with shrimp ferritin, suppresses apoferritin's ability to sequester free iron ions, and to counteract the host's iron withholding defense mechanism [52]. All these studies indicated ferritin's potential functions in immune response to WSSV infection. However, the specific mechanism of antivirus activity need further more research.

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