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A *CqFerritin* protein inhibits white spot syndrome virus infection via regulating iron ions in red claw crayfish *Cherax quadricarinatus*

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ABSTRACT

It is well known that iron is an essential element for all living organism. The intracellular iron availability is also important for the host's innate immune response to various pathogens, in which the iron homeostasis can be regulated by ferritin due to its iron storage property. In this study, a full-length cDNA sequence of ferritin (named as *CqFerritin*) was identified with 1410 bp from red claw crayfish *Cherax quadricarinatus*, which contained an open reading frame of 513 bp, encoding 170 amino acids with a conserved ferritin domain. Tissue distribution analysis demonstrated that *CqFerritin* was widely expressed in various tissues with high presence in haemocyte, haematopoietic tissue (Hpt) and heart, while lowest expression in hepatopancreas. In addition, loss-of-function of *CqFerritin* by gene silencing resulted in significantly higher expression of an envelope protein VP28 of white spot syndrome virus (WSSV) in red claw crayfish Hpt cell cultures, indicating the potential antiviral response of *CqFerritin*. To further explore the effect on WSSV replication by *CqFerritin*, recombinant *CqFerritin* protein (rCqFerritin) was transfected into Hpt cells followed by WSSV infection. Importantly, the replication of WSSV was obviously decreased in Hpt cells if transfected with rCqFerritin protein, suggesting that *CqFerritin* had clearly negative effect on WSSV infection. Furthermore, intracellular accumulation of iron ions was found to promote the WSSV replication in a dose-dependent manner, illustrating that the iron level regulated by *CqFerritin* was likely to be vital for WSSV infection in red claw crayfish. Taken together, these data suggest that *CqFerritin* plays an important role in immune defense against WSSV infection in a crustacean *C. quadricarinatus*.

1. Introduction

In the past few decades, white spot syndrome virus (WSSV) has broken out in the world and become a lethal pathogen for crustacean aquaculture, including shrimp and crayfish, and caused high mortality and huge economic loss (Escobedo-Bonilla et al., 2008; Lightner, 2011). Thus, it is crucial to find the efficient way to prevent and control this disease, and one approach to resolve the devastating pathogenic problem is to reveal the immune defense mechanism of crustaceans such as shrimp and crayfish against viral infection (Liu et al., 2009). WSSV is a large double-stranded DNA virus in which the viral genome contains gene encoding ribonucleotide reductase (RR), an essential enzyme required for DNA synthesis and repair (Tsai et al., 2000), and its activity relies on a metallo-cofactor binding with iron (Makhlynets et al., 2014). The insufficiency of cellular iron can restrain the activity of RR thus preventing new DNA synthesis and inhibiting cell proliferation (Cooper et al., 1996).

Ferritin, one of the major non-haem iron storage protein, was first reported in *Philaenus spumarius* in 1988 by Collin (Collin et al., 1988). The first decapod ferritin was identified from signal crayfish *Pacifastacus leniusculus* in 1996 (Huang et al., 1996). Soon after, it has been identified in a wide range of organisms from prokaryotes to eukaryotes (Theil, 2012). In vertebrates, ferritin is a globular protein complex consisting of 12 heavy and 12 light type subunits to form a nanocage with multiple metal–protein interactions,

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which can bind about 4500 iron atoms (Harrison and Arosio, 1996). While in plants and prokaryotes it is composed of 24 equal subunits (Theil, 1987). Ferritin has many important roles in a variety of biological processes, including iron storage source to prevent iron deficiency (Zielińska-Dawidziak, 2015), detoxification capacity and resistance against oxidative stress (Orino et al., 2001), as an important inflammatory marker of human disease (Kell and Pretorius, 2014), and heart protection by ischemic preconditioning via regulation of iron (Chevion et al., 2008). It also sequesters iron in the plasma, therefore reducing its availability to pathogens and prohibiting infection of pathogens (Ong et al., 2006).

To date, many researchers have confirmed the relationship between ferritin expression and host defense responses. For instance, the ferritin level was up-regulated in hepatitis C virus (HCV) infected patients (Oguz et al., 2013). Furthermore, an obvious increase in *ferritin* gene transcription was detected in the *Hyriopsis schlegelii* when stimulated with bacteria *Staphylococcus aureus* and *Vibrio anguillarum* (Sheng et al., 2016). In addition, up-regulation of ferritin had also been observed in WSSV-infected *Marsupenaeus japonicas* (Feng et al., 2014) and in polyriboinosinic polyribocytidylic acid (ploy (I:C)) or lipopolysaccharides (LPS) treated *Stichopus monotuberculatus* (Ren et al., 2014). Since ferritin functions on iron homeostasis and iron is an essential nutrient for most invading pathogens, the increase of ferritin level may constitute a defense mechanism against infection by deleting excessive free iron from the intracellular environment (Hu et al., 2010).

Previously, we found that *CqFerritin* transcript was up-regulated from a suppression subtractive hybridization library constructed from the haematopoietic tissue (Hpt) cell cultures of red claw crayfish *Cherax quadricarinatus* post WSSV challenge (Liu et al., 2011). Many studies have indicated that red claw crayfish can be infected by WSSV (Li et al., 2017; Wu et al., 2015) and crayfish Hpt cells have been demonstrated to be a good model for investigating the mechanism of WSSV infection and host defense (Chen et al., 2016; Jeswin et al., 2016; Liu et al., 2011). To elucidate the role of ferritin in WSSV infection, in the present study, we obtained the full-length cDNA sequence of *CqFerritin* and detected its expression profile in various tissues. Then the effect on WSSV replication in *CqFerritin* silenced Hpt cells or rCqFerritin overloaded Hpt cells by protein transfection were further investigated. Furthermore, the effect on WSSV infection by increasing intracellular iron was also examined. Our results found that *CqFerritin* exhibited strong inhibition effect on WSSV infection, which shed new light on *CqFerritin* function in innate immunity in a crustacean and provided useful strategy for white spot disease control.

2. Materials and methods

2.1. Animals, tissue collection and Hpt cell culture

Healthy red claw crayfish *C. quadricarinatus* (average weight 50±5 g) were purchased from the Yuansentai Technology Co. Ltd, Zhangzhou, Fujian Province and acclimatized in aerated freshwater at 26 °C for at least one week. Intermolt crayfish tissues or cells were collected. Haemocyte was obtained with a sterile syringe and centrifuged for 10 min with 1000 × g at 4 °C. Other tissues including Hpt, epithelium, gill, stomach, intestine, hepatopancreas, heart, gonad, muscle, nerve and eyestalk were sampled from three random individuals for total RNA isolation.

Hpt was isolated from *C. quadricarinatus* and the cells were cultured as described by Söderhäll (2013) with modification. Briefly, Hpt was dissociated into single cells with 600 μL collagenase mixtures, incubated at room temperature for 50 min. The tissue was washed once in 1 mL of crayfish phosphate buffered saline (CPBS) by centrifugation at 600 × g for 3 min to remove the collagenase solution. After resuspension with 1 mL of CPBS, cells were collected by centrifugation at 600 × g for 3 min and resuspended in L-15 culture medium, then cultured with 1.0×10^5 cells in 96-well plates and 5.0×10^5 cells in 24-well plates.

2.2. Total RNA extraction and cDNA synthesis

Total RNA from twelve tissues as described above was extracted with Trizol regent (Roche, Mannheim, Germany) according to the manufacturer's protocols. The extracted RNA was treated with RNase-Free DNase I (Ambion, Austin, Texas, USA) to eliminate genomic DNA contamination and resuspended in nuclease-free water. The total RNA concentration was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and the quality was estimated by 1.0% agarose gel electrophoresis. Total RNA (1 μg) was used for first strand cDNA synthesis using the PrimeScript™ RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Besides, total RNA extracted from Hpt was reversely transcribed using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Madison, Wisconsin, USA) for full-length cDNA obtained.

2.3. Gene cloning of CqFerritin full length cDNA

The open reading frame (ORF) of *CqFerritin* sequence was isolated from the transcriptome library of Hpt cells post WSSV infection in our lab (unpublished data) and was successfully amplified using the Fer-F and Fer-R primers (Table 1). Two gene-specific primers, Fer-5′RACE and Fer-3′RACE, were designed to obtain the 5′ and 3′ terminus of *CqFerritin* cDNA sequences by rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR). The specific primers and Universal Primer Mix (UPM) were shown in Table 1. Cycling conditions were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 65 °C for 30 s (decrease 0.3 °C per cycle); 72 °C for 2 min and 72 °C for 10 min. All the PCR productions were gel-purified using a Gel Extraction Kit (Sangon Bioteach, Shanghai, China) and cloned into the pMD18-T vector (TaKaRa, Japan). The vectors were transformed into *Escherichia coli* DH5α cells. Positive clones containing inserts of an expected size were sequenced at Borui Biotech Company, Xiamen, China.

2.4. Bioinformatics analysis

The ORF of *CqFerritin* was predicted using program ORF Finder in NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). The deduced amino acid sequence of *CqFerritin* was analyzed with simple modular architecture research tool, SMART (http://smart.embl-heidelberg.de/). Signal peptide was identified by SignalP 4.1

Table 1
Primer sequences for PCR amplification in this study.

Primers	Sequence
Fer-F	CGCGGATCCATGGCTTCCAGTGTCCGCCA
Fer-R	ACGCGTCGACCTATAGTAAATCTTTATCAAATATATGCAA
Fer-5′RACE	CCTGGAAGTGCCACATCATCTCTA
Fer-3′RACE	TGAAACAATAAAGAAACTTGGGGAC
UPM	CTAATACGACTCACTATAGGGC
dsFer-F	TAATACGACTCACTATAGGGTTCCAAGCAAGAATGGGATAAGG
dsFer-R	TAATACGACTCACTATAGGGATGAATCGTTACTTCCCTATTCTAGC
dsGFP-F	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT
dsGFP-R	TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC
qFer-F	GGCACTTCCAGGACTCTCCAAAT
qFer-R	TGCCCTCCAGTCCCTTATCCC
16S-F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA

Server (http://www.cbs.dtu.dk/services/SignalP/). The 3D domain structure of *CqFerritin* protein was conducted using SWISS-MODEL server (https://swissmodel.expasy.org/). Multiple sequences alignment was performed using the DNAMAN 6.0.3 program. The phylogenetic tree was constructed with Mega 6.06 software using the Maximum-likelihood method, based on the deduced amino acid sequence of *CqFerritin* with other homologous amino acid sequences in typical species from invertebrates to vertebrates and bootstrapped for 1000 times.

2.5. Expression profile analysis of CqFerritin in different tissues

The level of *CqFerritin* gene transcription in different tissues was examined by semi-quantitative real-time PCR (RT-PCR). The specific primers qFer-F and qFer-R (Table 1) were used to amplify target fragment and the crayfish 16S ribosomal gene (Genbank: AF135975.1) was used as the internal reference. Amplification was performed in a 20-μL reaction with 1 μL primer pairs (10 μM), 8 μL sterile water and 10 μL Taq PCR Mix (2 ×) (Dongsheng Biotech, Co Ltd, Guangzhou, China), 1 μL of DNA for target fragment or 50 times diluted cDNA for 16S group as template. The reaction program was 94 °C for 5 min, followed by 30 cycles of 10 s at 98 °C, 58 °C for 30 s, 72 °C for 30 s and 72 °C for 10 min. PCR assays were performed for three times. The PCR productions were analyzed by 1.2% agarose gel electrophoresis. The gray intensity values of the target gene bands, which were normalized to the level of 16S gene expression in the same sample, were measured by Gel Image System ID 4.2 program. The experiments were performed with three independently biological samples.

2.6. RNA interference assay of CqFerritin

To knockdown *CqFerritin* gene, an RNA interference assay was performed by the transfection of double-strand RNA (dsRNA) into Hpt cell cultures. The DNA templates of *CqFerritin* and green fluorescent protein (GFP) which served as the control were produced using the specific primers with a T7 promoter sequence at the 5′ terminus (Table 1). The dsRNA was synthesized using the MegaScript kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Before transfection into Hpt cells, dsRNA was verified by treatment with RNaseA.

The dsRNA was transfected into Hpt cell cultures according to the methods reported by Chen (Chen et al., 2016). Briefly, 100 ng dsRNA/well (96-well plates) or 400 ng of dsRNA/well (24-well plates) in RNase-free water was mixed with Cellfectin II Reagent (Life Technologies) and incubated for 10 min at room temperature, then the mixture was supplied with medium and added into the cell wells. The dsRNA transfection was repeated once one day after the first transfection to improve RNA interference efficiency. At 12 h after the second transfection, WSSV (MOI = 10) was added into 96-well plates cell cultures and incubated for 1 h followed by washed once with CPBS. The samples were collected at 12 h and 24 h after WSSV infection to detect the viral replication by immunoblotting against the viral envelope protein VP28 with β-actin as the internal reference. The experiments were repeated three times and the band intensities of VP28 were analyzed by Gel Image System ID 4.2 program. Meanwhile, the cells in 24-well plates were used to determine the interference efficiency of *CqFerritin* dsRNA in mRNA level.

2.7. Recombinant expression and purification of CqFerritin and TRX protein

The full length *CqFerritin* coding region was amplified by PCR with Fer-F and Fer-R (Table 1) with restriction enzyme cutting sites of *Bam*H I and *Sal* I. The PCR product was digested with *Bam*H I and *Sal* I and cloned into pET32a (+) to construct the expression vector pET32a-CqFerritin followed by transformation into *E. coli* BL21 (DE3) cells. After induction with 0.1 mM isopropy-β-D-thiogalactoside (IPTG), the recombinant protein of *CqFerritin* was expressed at 28 °C for 8 h. The pET32a vector without insert sequence was used as a control expressing rTRX protein.

The bacteria were resuspended in cold lysis buffer and sonicated. The lysate was centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was mixed gently with 1 mL of Ni Fesin FF (Genscript, USA) beads for 1 h at 4 °C. After rinsing with ice-cold PBS containing 10 mM imidazole, the fusion protein was eluted with PBS containing 100 mM imidazole and dialysed in 20 mM HEPES buffer for 48 h. The purified protein was analyzed by SDS-PAGE and the concentration of purified protein was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

2.8. Transfection of rCqFerritin recombinant protein

The Hpt cell cultures in 96-well plates were transfected with ferritin recombinant protein delivered by PULSin reagent (Polyplus, France), according to the manufacturer's instructions. Briefly, 300 ng of rCqFerritin protein was diluted in 20 mM HEPES of final volume of 20 μL and vortexed gently followed by the addition of PULSin and incubated at room temperature for 15 min. The mixture was then added into the cell cultures and incubated for 4 h followed by the WSSV (MOI = 10) infection. After 1 h infection, the cell cultures were washed once with CPBS. Finally, cells were collected at 3, 6, 12 and 24 h post infection (hpi), respectively, for the determination of viral VP28 with Western blotting. Cells treated with rTRX protein were used as the control.

2.9. Hpt cells treated with ferric ammonium citrate (FAC)

After separated from hematopoietic tissue, the Hpt cells were cultured with modified L-15 medium for at least 3 h in 96-well plates. Then the Hpt cell cultures were treated with 100 μM or 200 μM FAC for 12 h at 20 °C. Same volume sterile water was used as a solvent control. The cell cultures were incubated with WSSV (MOI = 10) for 1 h followed by washing once with CPBS, then the medium containing iron and WSSV was replaced with fresh L-15 medium and cultured for 6 and 12 hpi, respectively, at 26 °C. The Hpt cell cultures were collected at corresponding time points with 1 × SDS loading buffer for the detection of viral VP28 by immunoblotting. The experiments were performed with three independently biological samples.

The protein samples collected from 96-well plates Hpt cells with 1 × SDS loading buffer were resolved by 12% SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. Then the membrane was blocked with 5% skim milk in TBST for 1 h at room temperature, and subsequently incubated with mouse anti-VP28 antisera (1:3000) or anti-β-actin antibody (1:3000) (TransGene Biotech, Beijing, China) for 2 h at room temperature. The membranes were then gently washed three times with TBST buffer, followed by incubation for 1 h at room temperature with HRP-conjugated goat anti-mouse secondary antibodies (1:5000). After brief wash, the bands were detected by immunoblotting.

2.10. Statistical analysis

All numerical data were analyzed by Student's *t*-test and presented as the mean ± standard deviation (SD) from more than three independent assays using the Statistical Product and Service Solutions (SPASS) package. Differences with $p < 0.05$ were considered as significant difference.

3. Results and discussion

3.1. Gene cloning and sequence analysis of CqFerritin

The full-length cDNA sequence of *CqFerritin* (Genbank: MG649969) was identified using RACE technology. As shown in Fig. 1, the full-length cDNA of *CqFerritin* was 1410 bp with a 5′-untranslated region (UTR) of 248 bp, a 3′-UTR of 649 bp and an ORF of 513 bp encoding 170 amino acids. The calculated molecular mass of the deduced *CqFerritin* protein was about 19.5 kDa with pI of 6.22. A putative Iron Responsive Element (IRE) was found based on the analysis of the 5′-UTR according to Durand's method (Durand et al., 2004), which contained a typical 5′-CAGTGN-3′ sequence to form a loop in the IRE mRNA secondary structure. This important regulatory element acts as a binding site for iron regulatory proteins (IRPs), which regulates the genes expression involved in iron metabolism (Hentze and Kuhn, 1996). In contrast, the ferritin mRNA of plants, yeast and bacteria do not contain this IRE element (Theil, 1987). As shown in Fig. 2A, the deduced amino acids of *CqFerritin* shared relatively high identities with those amino acids of other species deposited in the Genbank database. Multiple alignments of the ferritin amino acid sequences from crustacean and fish indicated the consensus sequences in ferritins. The homology analysis showed that *CqFerritin* shared the highest identity (57.06%) with the crab ferritin from *Scylla paramamosain* and less from other species, including *Litopenaeus vannamei* (56.47%), *Eriocheir sinensis* (54.71%) and *Danio rerio* (48.59%).

The domain architecture prediction by SMART showed that the deduced *CqFerritin* protein contained a conserved ferritin domain (residues 14–155) (Fig. 2B) which possessed typical ferritin features including ferroxidase diiron center, ferrihydrite nucleation center and iron ion channel. No signal peptide was found in *CqFerritin* protein by SignalP analysis prediction, indicating that *CqFerritin* protein was present intracellularly but not extracellularly in red claw crayfish. However, a signal peptide for protein secretion was found in the N-terminal of *Galleria mellonella* ferritin which was proposed to serve as a crucial molecule for iron-withholding defense response in serum (Kim et al., 2002). The 3D structure of *CqFerritin* was estimated to be a globular protein complex consisting of 24 protein subunits (Fig. 2C), in which each protein subunit contained four long helices and one short helix (Fig. 2D) similar to that of ferritin from *Procambarus clarkia* (Liu et al., 2017). To figure out the evolutionary position of ferritin, a phylogenetic tree was constructed using *CqFerritin* amino acid sequence and other homologous of typical species by the Maximum-likelihood method. As shown in Fig. 3, *CqFerritin* was mostly clustered with crustacean ferritin, fish heavy type ferritin, *P. leniusculus* ferritin and mammal light chain subunits. However, within this clade, *CqFerritin* formed a separate branch by itself. In contrast, the overall sequence similarities among vertebrate heavy type ferritins were comparatively lower. The characteristic of *CqFerritin* phylogenetic tree was consistent with *M. japonicas* which shared relatively high identity with crab and shrimp (Feng et al., 2014). These data together illustrated the molecular character of the *CqFerritin* gene which provides new light on the ferritin function in a crustacean.

3.2. Tissue distribution of CqFerritin

Semi-quantitative RT-PCR was performed to determine the relative expression of *CqFerritin* in different tissues of red claw crayfish, including Hpt, haemocyte, heart, hepatopancreas, stomach, intestine, muscle, nerve, epithelium, gonad, gill and eyestalk.

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1       ACATGGGGGAGTGAGGACGACCAGCACTGTCAGCCAGCACTGTCAGCTGCTTCCTGTCACCACCTTCACTTCACCCAGGAAGGATATAGT
91      CAACATCAGTGATACAACAGCCTGGATATAGTGAGAGTCGGTAATATAGCAGCCTGGATATAGTGAGAGTCGGTAATATAGCAGCCTGGA
181     TATAGTGAGAGTCGGTAATATAGCAGCCTGAATATAGTGAGAGTCAGTAATATAGGAGCAGCGTGAAGATGGCTTCCAGTGTCCGCCACA
1                                                                       M  A  S  S  V  R  H
271     ACTATCACGAAGACAATGAAGCTGCTCTCAACAAATATATTAACCTGGAACTACATGCCAGCTATGTCTTCTTGGCCTTGAGCTACCACT
8       N  Y  H  E  D  N  E  A  A  L  N  K  Y  I  N  L  E  L  H  A  S  Y  V  F  L  A  L  S  Y  H
361     TTGATAGAGATGATGTGGCACTTCCAGGACTCTCCAAATTGTTCAGAGGCTACAGTGACTTTGAGCTTGTTAATGCACACAAGTTAATGA
38      F  D  R  D  D  V  A  L  P  G  L  S  K  L  F  R  G  Y  S  D  F  E  L  V  N  A  H  K  L  M
451     AGTACCAGAACCAGCGTGGTGGCCGGGTCGTTCTTCATGACGTATTTCCTCCTTCCAAGCAAGAATGGGATAAGGGACTGGAGGGCATTC
68      K  Y  Q  N  Q  R  G  G  R  V  V  L  H  D  V  F  P  P  S  K  Q  E  W  D  K  G  L  E  G  I
541     AGACAGCTTTAGATCTGAAGAAAGAACTTAATGAGGCCCTGCTAAATCTTCATGGAAAGGTATCTGAAACGAATGATCCCCATGTTCTCC
98      Q  T  A  L  D  L  K  K  E  L  N  E  A  L  L  N  L  H  G  K  V  S  E  T  N  D  P  H  V  L
631     ATTTCTTGGATGATAATTTCATAAATGAGCATGTTGAAACAATAAAGAAACTTGGGGACATGGTGACACAGCTCCAACGTGCAGGAGACG
128     H  F  L  D  D  N  F  I  N  E  H  V  E  T  I  K  K  L  G  D  M  V  T  Q  L  Q  R  A  G  D
721     GCCATCTTGGTTTGCATATATTTGATAAAGATTTACTATAGTTTGGAAATGATAGAAAAATGTGTTGGGCTTTTGGCTAACGTTTGGTCT
158     G  H  L  G  L  H  I  F  D  K  D  L  L  *
811     TGACAACTGTGGTATGTCTTTAGTAATATTAATCATGTTGAGCTACTGTATCTTTTTGTCATATATTCCTTTTTTTGTTGTAGAATTTCT
901     TAATGTGTTATGTTTTAAGCTTCTTGTGTAGCACATTATCTTATCACTTAAAAGCTAGAATAGGGAAGTAACGATTCATATACTGACACT
991     TAAATTACTGTGTAGAATTGTGGATAGTTAATTTGTAAATTTGTAATTTAGCATTTTTTAATGAAATTATATTTTACTGTAGCAATATGT
1081    AATATCTTTATTACTGACTTCTGTCACTGATGTGGTTTGTGTAAATTGATTTTTCCTTGCAAGCTCAAATGTTAATTGTACTTAATACTC
1171    AGACACTTAAATCTTATAGTAATGATTGTACTTTTGACTATAGCATCTTCTATGCACATTTATAATAAACTACCTTTTCAGAAAGGATGG
1261    GATTCAAACCCATGGCAAGCCAGTCCTAAAACTGGACTATGCCACCGTGGGTTAGAATCCTGATGTCTGAAAGTTGTTGCAAAATATCAA
1351    AACAATTGTGAGTTTATAGTAAGGCAAAAGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of *CqFerritin*. Nucleotides and amino acids were numbered on the left of the sequence. The box denoted the start codon (ATG) and the stop codon (TAG). The conversed ferritin domain was underlined and the IRE binding region was marked with double lines.

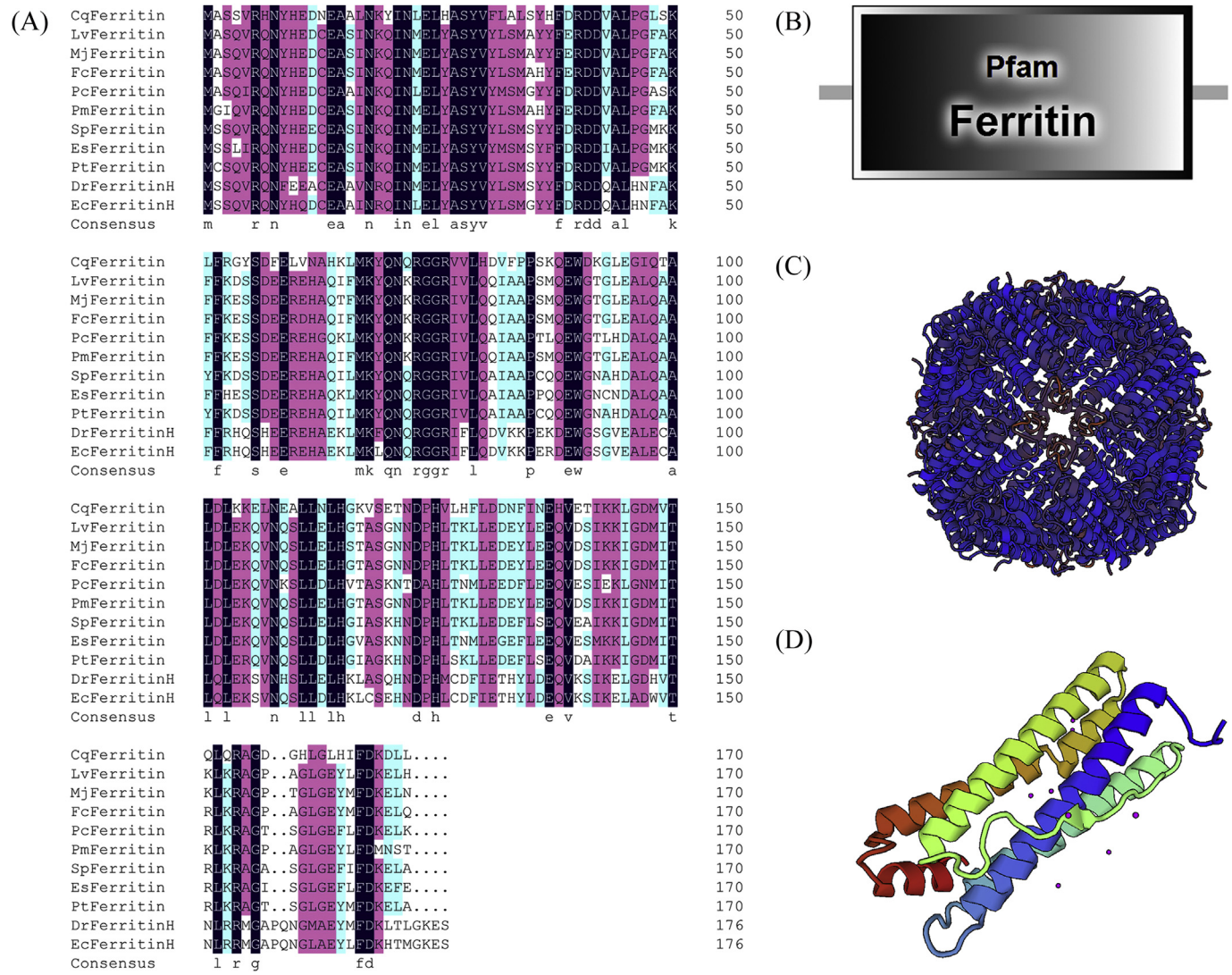

Fig. 2. The bioinformatics analysis of *CqFerritin* sequence. (A) Multiple sequences alignment of *CqFerritin*. Conserved amino acids were in colors. The amino acids sequences of ferritin were from *C. quadricarinatus* and other species including *L. vannamei*, *Marsupenaeus japonicus*, *Fenneropenaeus chinensis*, *Procambarus clarkia*, *Penaeus monodon*, *S. paramamosain*, *E. sinensis*, *Portunus trituberculatus*, *D. rerio* and *Epinephelus coioides*. (B) Predicted protein domain structure of *CqFerritin*. The *CqFerritin* protein contained a conserved domain of ferritin. (C) The 3D structure model of *CqFerritin* protein with 24 equal subunits. (D) The 3D structure model of one subunit from *CqFerritin* protein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The result revealed that the mRNA of *CqFerritin* was detected in all tested tissues (Fig. 4) with higher expression in haemocyte, Hpt and heart followed by less expression in gill, intestine and nerve. This expression profile was in consistent with that of *L. vannamei* ferritin (Hsieh et al., 2006). Meanwhile, *CqFerritin* had lower expression in stomach, muscle, gonad, eyestalk and epithelium, and lowest expression in hepatopancreas. In contrast, few ferritin mRNA transcript was detected in haemocyte from the freshwater giant prawn, ridgetail white prawn and red swamp crayfish, while high amounts of transcripts were found in the hepatopancreas (Liu et al., 2017; Qiu et al., 2008; Zhang et al., 2015). It has been reported that ferritin mRNA exhibits tissue specific expression patterns in various animal species. In consideration to that ferritin is a universal intracellular protein that stores and releases iron in a controlled pattern, its expression profile depends on the iron storage capacity of each tissue. Hence, such a wide range distribution of ferritin indicated that *CqFerritin* was likely to play an important role in iron homeostasis in red claw crayfish. It is well known that crustacean is lacking of adaptive immunity and haemocyte plays a critical role in innate immune response against pathogens. Besides, haemocyte is the major metabolic center for the production of reactive oxygen and ferritin is responsible for sequestering excess iron and dioxygen reaction products against the oxidant damage in crustaceans (Huang and Xu, 2016; van de Braak et al., 2002). Therefore, the highest expression of *CqFerritin* in the haemocyte strongly indicated that *CqFerritin* was likely to be associated with immune response in red claw crayfish.

3.3. Increased WSSV replication by gene silencing of CqFerritin in crayfish Hpt cell cultures

Previously, the transcript of ferritin was reported to be up-regulated post WSSV challenge in both red claw crayfish Hpt (Liu et al., 2011) and shrimp (Ye et al., 2015), clearly indicating that *CqFerritin* might be involved in host immune defense against WSSV infection. To reveal whether *CqFerritin* affected on WSSV infection, the expression of *CqFerritin* gene was knocked down in red claw crayfish Hpt cell cultures followed by WSSV infection. As shown in

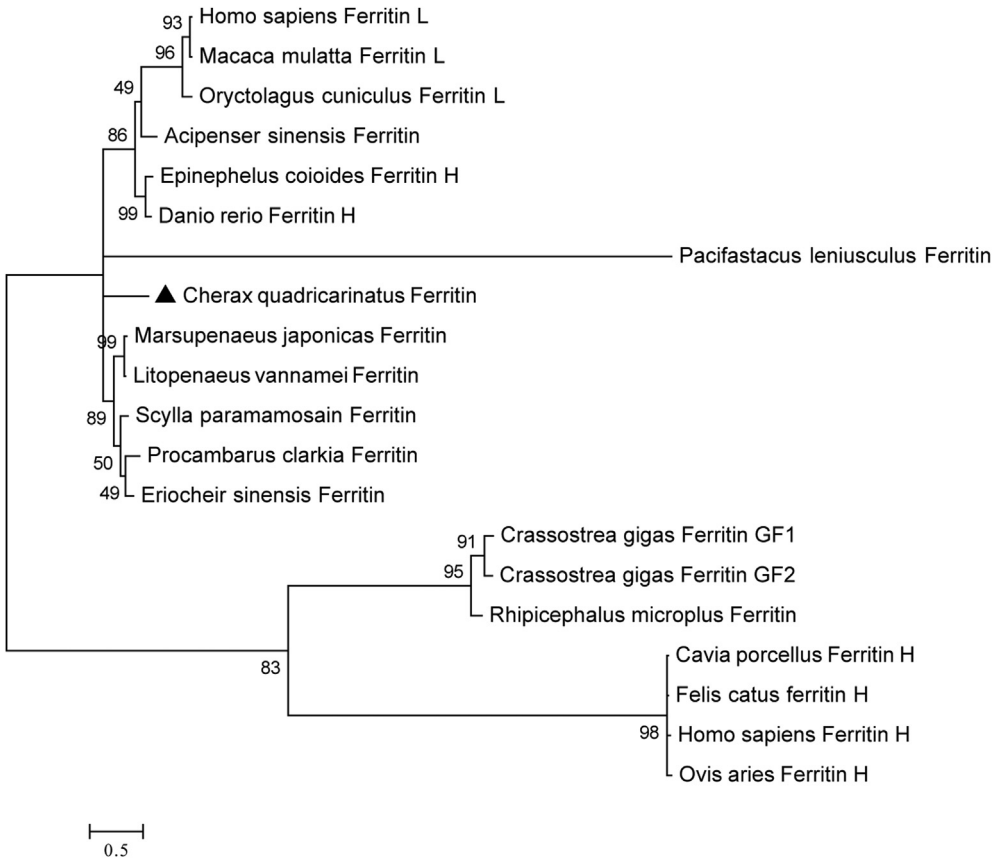

Fig. 3. The phylogenetic tree of ferritins from invertebrates and vertebrates. *CqFerritin* was marked with black triangle. The Genbank ID of sequences were shown as follows: *Homo sapiens H* (AAA35832.1); *Ovi saries H* (NP_001009786.2); *Felis catus H* (BAE78405.1); *Cavia porcellus H* (BAB70615.1); *Homo sapiens* L (NP_000137.2); *Macaca mulatta* L (NP_001248136.1); *Oryctolagus cuniculus* L (NP_001095158.1); *Epinephelus coioides H* (AEG78374.1); *D. rerio H* (NP_571660.1); *Acipenser sinensis* (ABY81252.1); *E. sinensis* (ADD17345.1); *P. clarkia* (AEB54659.1); *S. paramamosain* (ADM26622.1); *L. vannamei* (AAX55641.1); *M. japonicus* (AGV07611.1); *Rhipicephalus microplus* (AAQ54710.1); *Crassostrea gigas* GF1 (AAP83793.1); *Crassostrea gigas* GF2 (AAP83794.1); *Pacifastacus leniusculus* (CAA62186.1).

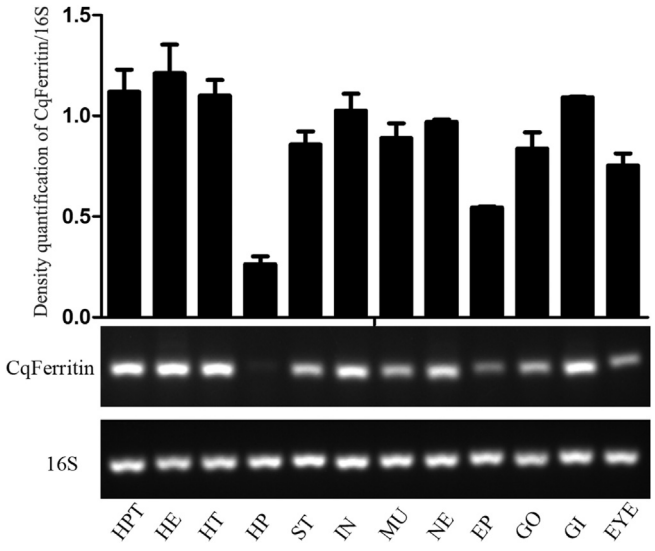

Fig. 4. Tissue distribution profile of *CqFerritin*. The expression of *CqFerritin* in different tissues was detected by semi-RT-PCR (lower panel). The band intensities of three independent experiments were calculated using Gel Image System ID 4.2 program (upper panel). The 16S rRNA was used as an internal control. HPT: hematopoietic tissue; HE: haemocyte; HT: heart; HP: hepatopancreas; ST: stomach; IN: intestine; MU: muscle; NE: nerve; EP: epithelium; GO: gonad; GI: gill; EYE: eyestalk.

Fig. 5A, the gene expression of *CqFerritin* was significantly reduced more than 99% at both 12 hpi and 24 hpi compared to GFP dsRNA treated control cells, suggesting that the *CqFerritin* was efficiently silenced in Hpt cells during WSSV infection. Meanwhile, the expression of a viral envelope protein VP28 was detected by Western blotting in *CqFerritin* silenced Hpt cells. The signal density quantification analysis showed that the expression of VP28 were relatively increased to 1.5-fold at both 12 h and 24 h post WSSV infection compared to the control treatment (Fig. 5B). Therefore, loss-of-function of *CqFerritin* gene led to the increase of viral replication, indicating that *CqFerritin* was associated with anti-WSSV response in crayfish Hpt cells. Similar result was found that knocking down of ferritin resulted in more susceptibility to WSSV in *L. vannamei* (Ye et al., 2015). Besides, *Scapharca broughtonii* ferritin has been reported to inhibit the growth of Gram-negative bacteria *E. coli* and Gram-positive bacterium *S. aureus* and *Micrococcus luteus*, in which it was speculated to interact with certain components of bacteria cell wall, or enter into bacterial cell directly, or function as part of an anti-oxidant response (Zheng et al., 2016). Thus, all the results indicated that ferritin possessed an important immune function in invertebrates.

3.4. Reduced WSSV replication by rCqFerritin transfection into crayfish Hpt cells

As shown above, gene silencing of *CqFerritin* resulted in increased WSSV replication. We then speculated that what would

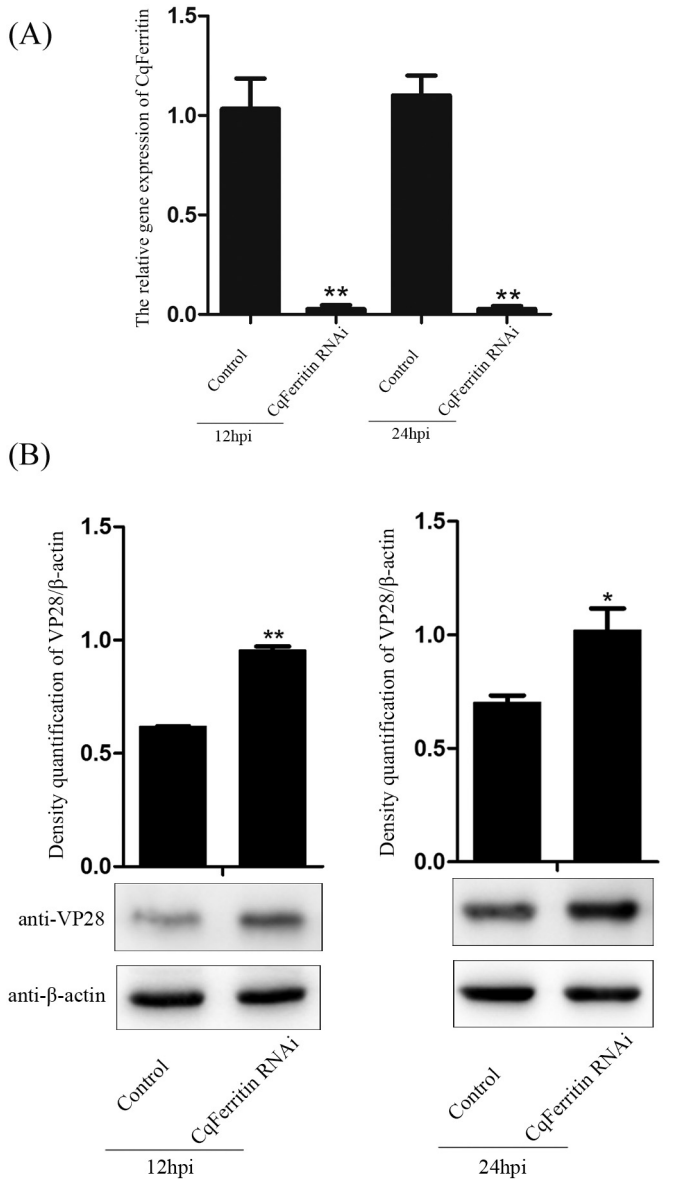

Fig. 5. Effect on WSSV replication by loss-of-function of *CqFerritin* gene in crayfish Hpt cells. (A) The mRNA expression of *CqFerritin* was determined by RT-PCR after gene silencing of *CqFerritin* during WSSV infection. The 16S rRNA was used as an internal control. (B) The expression of viral VP28 was detected at 12 hpi and 24 hpi after gene silencing of *CqFerritin* by immunoblotting (lower panel). The band intensities of three independent experiments were calculated by using Gel Image SystemID 4.2 program (upper panel). GFP dsRNA treatment was used as the control groups. The asterisk indicated significant difference compared with those of controls ($^{*}p < 0.05$, $^{**}p < 0.01$).

happen to the WSSV infection if rCqFerritin protein was artificially overloaded in Hpt cells. The rCqFerritin was expressed with His-TRX-tag using a prokaryotic expression system and further purified via Ni Resin FF beads. The rTRX with a His-tag was also expressed and purified as a control protein. As shown in Fig. 6, rCqFerritin protein was observed at approximately 37 kDa, which was in agreement with the predicted molecular weight of 19.5 kDa since the His-rTRX protein was 18 kDa. The molecular weight of rCqFerritin was similar to that of other crustaceans like *Fenneropenaeus chinensis* (Zhang et al., 2006), *L. vannamei* (Ye et al., 2015) and *Macrobrachium nipponense* (Sun et al., 2014). Besides, the rCqFerritin protein was confirmed by MALDI-TOF/TOF mass spectrometry analysis, which demonstrated that the peptide fragments

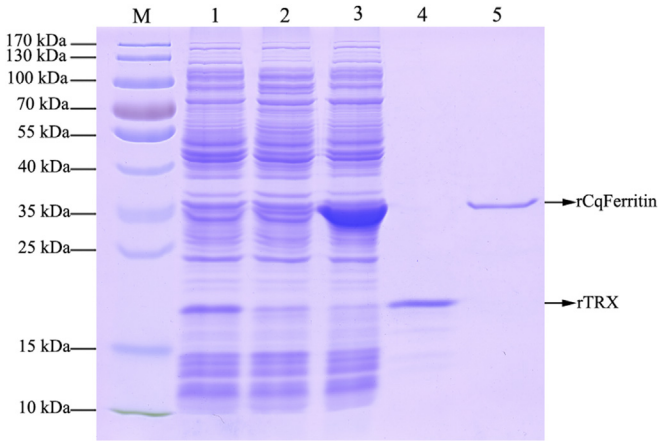

Fig. 6. Expression and purification of rCqFerritin protein. Lane M: protein molecular standard; 1: pET32a-BL21 recombinant clone, IPTG induced; 2: pET32a-BL21 recombinant clone containing *CqFerritin* gene, non-induced; 3: pET32a-BL21 recombinant clone containing *CqFerritin* gene, IPTG induced; 4: purified recombinant TRX protein; 7: purified rCqFerritin protein.

were in correspondence to the deduced amino acids of *CqFerritin* (data not shown). Besides, the purity of both two recombinant proteins was more than 90% and suitable for protein transfection.

To further elucidate the effect on WSSV replication by overloading of rCqFerritin, the rCqFerritin protein was transfected into Hpt cells followed by WSSV infection at 4 h post protein transfection. The results showed that the expression of viral VP28 was significantly lower in rCqFerritin transfected cells than that of control groups during the time periods tested (Fig. 7). These data indicated that overloaded ferritin protein inhibited WSSV replication in protein level probably by depriving the iron ions necessary for WSSV infection. In addition, ferritin was also reported to participate in anti-pathogen response in shrimp. For instance, the survival rate of *L. vannamei* injected with ferritin protein was significantly higher than that of saline control animals during

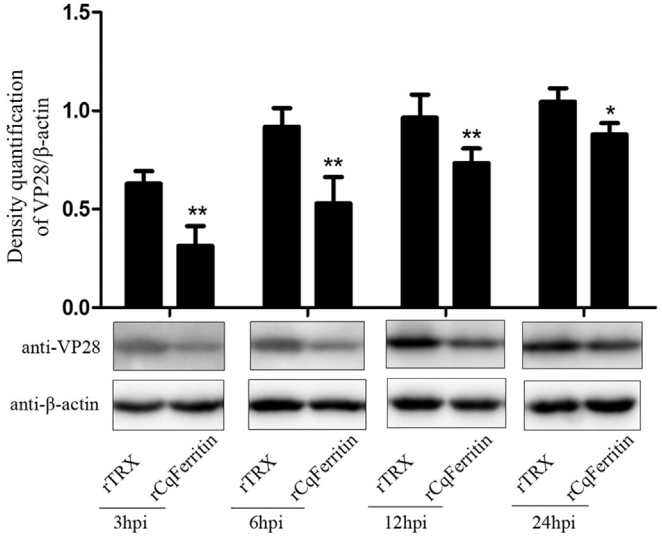

Fig. 7. Inhibition on WSSV replication by overloading rCqFerritin protein into Hpt cells. The expression of viral VP28 and β-actin were determined by Western blotting after transfection of rCqFerritin protein into Hpt cells. The replication of WSSV in rCqFerritin treated groups was significantly reduced compared to the control groups accordingly. rTRX protein was used as the control groups. This experiment was completed in biological triplicates.

WSSV infection (Ruan et al., 2010). Furthermore, injection of recombinant ferritin reduced the mortality in *P. monodon* infected with *Vibrio harveyi*. However, the recombinant ferritin did not show any direct antimicrobial activity against *V. harveyi*, and its addition did not reduce viable counts of *V. harveyi*. Hence, the protective activity of ferritin protein was probably by restricting the availability of iron in haemocytes of black tiger shrimp (Maiti et al., 2010). Taken together, the clearly negative effect on WSSV replication by overloaded rCqFerritin was possibly due to its iron chelating property but which needs furthermore investigations.

3.5. Increased WSSV replication by intracellular accumulation of iron ion

As an iron storage protein, ferritin plays a key role in iron metabolism in which iron is an essential nutrient for most organisms including pathogens (De Zoysa and Lee, 2007). Iron serves as a co-factor in many enzymes like ribonucleotide reductase. In particular, iron is extremely required for ribonucleotide reductase activity which is critical for the replication of DNA viruses such as WSSV (Cooper et al., 1996; Lin et al., 2015). Hence, the level of intracellular iron ions which could be regulated by ferritin was likely to impact the replication of WSSV. To determine whether changes in iron availability have any impact on WSSV replication in crayfish Hpt cell cultures, the cells were pretreated with FAC followed by WSSV infection. As shown in Fig. 8, the expression of viral VP28 was significantly increased in a dose-dependent manner in FAC treated cells compared with control groups at both 6 h and 12 h post viral infection, indicating that intracellular accumulation of iron had clearly positive effect on WSSV replication. Previous study also indicated that rising iron availability with FAC promoted the replication of West Nile virus (WNV), while treating with iron chelator (deferoxamine) inhibited the viral replication. The regulation of WNV infection was mainly resulted from the IREs response mediated by iron availability (Duchemin and Paradkar, 2017). Similarly, reduced availability of iron inhibited viral replication like human immunodeficiency virus type 1 (Georgiou et al., 2000), vaccinia and herpes simplex virus (Romeo et al., 2001). Moreover,

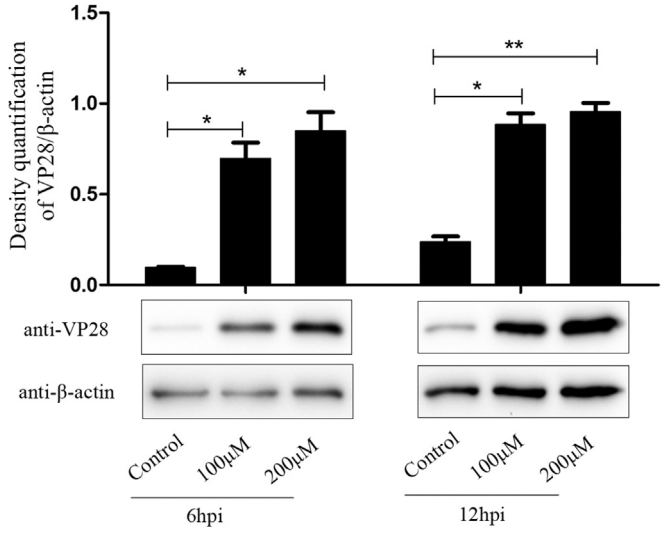

Fig. 8. Increased WSSV replication with excessive FAC. Hpt cells were pretreated with FAC (0, 100 μM, 200 μM) for 12 h, respectively, followed by infection with WSSV (MOI = 10), then the cells were correspondingly harvested at 6 hpi and 12 hpi. The viral envelope protein VP28 and reference protein crayfish β-actin were detected by immunoblotting (lower panel). The band intensities were analyzed by using Gel Image System ID 4.2 program (upper panel). This experiment was repeated for three times.

iron could promote the translation of HCV by stimulating the expression of eukaryotic initiation factor 3 (eIF3), which is an indispensable factor for effective initiation of HCV RNA translation (Theurl et al., 2004). However, other researches confirmed that supra-physiological intracellular iron induced by haemin treatment could reduce HCV replication of the full genome level (Bartolomei et al., 2011). All these data together indicated that the intracellular iron level has a key impact on virus replication, but the exact mechanism of iron requirement in WSSV replication is not so clear.

4. Conclusion

In summary, a ferritin gene *CqFerritin* was identified from red claw crayfish *C. quadricarinatus*. Functional study revealed that *CqFerritin* played a critical role in anti-WSSV response in a crustacean, which was likely to be due to the deprivation of iron ions by *CqFerritin* since iron was essential for virus replication. Therefore, these data will be helpful for the further mechanism elucidation of anti-WSSV response, new thoughts of WSSV disease control, and better selection of feed additives in crustacean farming.

Acknowledgement

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