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A thymosin repeated protein1 reduces white spot syndrome virus replication in red claw crayfish *Cherax quadricarinatus*

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ABSTRACT

The β-thymosins are a group of structurally related, highly conserved intracellular small peptides in vertebrates with various biological functions, including cytoskeletal remodeling, neuronal development, cell migration, cell survival, tissue repair and inhibition of inflammation. In contrast to vertebrates, the function of β-thymosin is not fully understood in crustaceans. Previously, we found that a thymosin-repeated protein1 (*CqTRP1*) gene was up-regulated after white spot syndrome virus (WSSV) challenge in hematopoietic tissue (Hpt) cells from the red claw crayfish *Cherax quadricarinatus*. To further identify the effect of CqTRP1 on WSSV infection, a full length cDNA sequence of β-thymosin homologue was cloned and analyzed from red claw crayfish followed by functional study. The *CqTRP1* cDNA contains an open reading frame of 387 nucleotides encoding a protein of 129 amino acids with a putative molecular mass of 14.3 kDa. The amino acid sequence showed high identity with other β-thymosins and contained three characteristic thymosin β actin-binding motifs, suggesting that *CqTRP1* was a member of the β-thymosin family. Tissue distribution analysis revealed a ubiquitous presence of *CqTRP1* in all the examined tissues with the highest expression in hemocytes, Hpt and gonad at the transcriptional level. Interestingly, the gene silencing of endogenous *CqTRP1* by RNAi enhanced the WSSV replication in Hpt cells. Meanwhile, the WSSV replication was significantly reduced in the Hpt cell cultures if overloaded with a recombinant CqTRP1. Taken together, these data clearly indicated that CqTRP1 was likely to be associated with the anti-WSSV response in a crustacean *C. quadricarinatus*, which provides new strategy against white spot disease in crustacean aquaculture.

1. Introduction

The white spot syndrome virus (WSSV) is one of the most serious pathogens which affect the cultivation of global economic shrimp species. In addition to the impact of the virus on the economy, the natural marine ecology is also threatened by this virus as WSSV is able to infect a large number of crustaceans including crabs and freshwater crayfish (Lo et al., 1996; Wang et al., 1998). The WSSV virions can be found in most tissues and can circulate in the hemolymph of the infected crustaceans (Witteveldt et al., 2004). It is well known that crustaceans are lacking of adaptive immune response and dependent solely on the innate immune response to recognize and destroy the invading exogenous pathogens (Lee and Söderhäll, 2002). The host defense mechanisms and particularly host anti-viral defense of crustacean are still poorly understood (Gross et al., 2001). Hence, it is important to have a better understanding of relationship between WSSV infection and the host innate immune factors which will benefit the novel control strategies against white spot disease.

Thymosin, a family of polypeptide hormones isolated from thymus tissue, has been found from calf thymus first (Huff et al., 2001). Furthermore, thymosins were named and classified according to its capacity to stimulate lymphocytopoiesis and their isoelectric points: α-thymosins with pI below 5.0, β-thymosins with pI range from 5.0 to 7.0, and γ-thymosins with pI above 7.0 (Huff et al., 2001). Thymosin-β4 (Tβ4) is the most abundant member and representing about 70–80% of the total β-thymosin content, with a 43 amino acid peptide and about 5 kDa molecular mass (Goldstein et al., 2005; Huff et al., 2001; Low and Goldstein,

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1982; Low et al., 1981). Importantly, Tβ4 is highly conserved from mammals to echinoderms and plays important role in development and immune response (Kang et al., 2011; Sanders et al., 1992; Stoeva et al., 1997; Zhang et al., 2008). To date, the β-thymosin-like genes have been reported in invertebrates like *Drosophila melanogaster* (Koshikawa et al., 2010), *Caenorhabditis elegans* (Van et al., 2004) and *Helicoverpa armigera* (Saelee et al., 2013) and *Hodotermopsis sjostedti* (Koshikawa et al., 2010). Furthermore, in aquaculture animals, the studies on β-thymosins are also limited to only a few species, such as Chinese mitten crab *Eriocheir sinensis* (Gai et al., 2009), red swamp crayfish *Procambarus clarkia* (Shi et al., 2015) and disk abalone *Haliotis discus discus* (Kasthuri et al., 2013). Most reports about β-thymosin in invertebrates are related to its ability to promote growth and resist pathogens invasion, even to regulate the proliferation and differentiation of hematopoietic stem cells. However, the knowledge about the roles of invertebrate β-thymosin is still largely unknown, especially in terms of anti-viral immunity.

Previously, we found that a partial sequence of thymosin-repeated protein1 (*CqTRP1*) was responsive to WSSV infection in red claw crayfish Hpt cells (Liu et al. 2011). To reveal the role of *CqTRP1* in WSSV infection, in the present study, we obtained the full-length cDNA sequence of *CqTRP1* and determined its gene expression profile in various tissues. Then the effect on WSSV replication in *CqTRP1* silenced hematopoietic tissue (Hpt) cells or r*CqTRP1* overloaded Hpt cells by protein transfection were further examined. Our results found that *CqTRP1* showed strong reduction on WSSV replication, which provided new information on *CqTRP1* function in antiviral immunity in a crustacean and further useful strategy for white spot disease control.

2. Materials and methods

2.1. Experimental animals and preparation of Hpt cells

The healthy freshwater red claw crayfish, *C. quadricarinatus*, were purchased from Source Sentai Agricultural Science and Technology Co., Ltd of Zhangzhou, Fujian Province, China, and kept in tanks in aerated tap water at 26 °C. The Hpt cells of crayfish were prepared and cultured according to Söderhäll et al. (2005). The hematopoietic tissue was dissected from the dorsal side of the stomach, and washed with CPBS (phosphate buffer saline of crayfish: 10 mM Na_2HPO_4; 10 mM KH_2PO_4; 150 mM NaCl; 10 mM $CaCl_2$ and 10 mM $MnCl_2$; pH 6.8) and then incubated in 500 μL of 0.1% collagenase (type I) and 0.1% collagenase (type IV) (Sigma) in CPBS at room temperature for 45 min. The Hpt was centrifuged at 800 × g for 3 min at room temperature and to remove the collagenase solution. The samples were washed twice with 1 mL CPBS in the same method described above and the cells were isolated by gently pipetting and to remove the undigested tissues, and then the isolated Hpt cells were resuspended in L-15 medium (Söderhäll et al., 2005). Hpt cells were seeded in a 24-well plates at a density of 5×10^5 cells/500 μL and supplemented with plasma (a crude astakine preparation from red claw crayfish) (Söderhäll et al., 2005) after about 30 min attachment at 20 °C.

2.2. Virus preparation

The WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China). The virus was prepared as described in the article of Xie et al. and quantified via absolute quantification by PCR (Xie et al., 2005).

2.3. RNA extraction and cDNA synthesis

Different tissues of crayfish were collected and total total RNA was extracted from all the tissues with TRIzol reagent (Roche, USA) according to the manufacturer's instructions. RNase-Free DNase I (Ambion, USA) was used to eliminate genome DNA contamination in the extracted RNA. The RNA samples were analyzed in 1.0% agarose electrophoresis and quantitated at 260 nm with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and all OD260/OD280 were between 1.8 and 2.0. 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. The 3′ and 5′ ends RACE cDNA templates were synthesized using SMARTer™ cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

2.4. Gene cloning of the full-length cDNA of CqTRP1

A partial *CqTRP1* cDNA sequence of *C. quadricarinatus* was obtained from a transcriptome library of Hpt cells post WSSV infection in our lab (Liu et al., 2011). BLAST analysis showed that this *Cherax β-thymosin* showed high level of identity (or similarity) with *β-thymosins* of other shrimps. Based on the transcriptome library sequence data of *CqTRP1*, its 3′ and 5′ ends were obtained using SMARTer™ cDNA Amplification Kit (Clontech, USA). For 3′ RACE, the PCR reaction was conducted using the primer F1 and the anchor primer UPM (Table 1). The PCR reaction conditions were 98 °C for 2 min, 30 cycles of 98 °C for 30 s, 65 °C for 30 s and 72 °C for 40 s, and 72 °C for 10 min. For 5′ RACE, as well as those described above.

All amplified PCR fragments were subjected to electrophoresis on 1.0% agarose gel to determine length differences, and the target spot was purified by PCR Gel Extraction Kit (Sengong Biotech, Co., Ltd., Shanghai, China). The final purified products were cloned into PMD18-T vector (TaKaRa, Japan), following the instructions provided by the manufacturer. The vectors were transformed into *E. coli* DH5α cells. Recombinant bacteria were confirmed by PCR and further sequenced at Xiamen Borui Biotech Company, China.

2.5. Sequence analysis and domain search analysis

The cDNA sequence of *CqTRP1* had been published previously (Genbank accession no: AEL23126.1). The nucleotide and deduced amino acid sequences of *CqTRP1* cDNA were analyzed and compared using the BLASTn and BLASTp search programs (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). A search for conserved domains and motifs was performed using the conserved domain database of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the prediction map from SMART (http://smart.emblheidelberg.de). The signal peptide was identified using SignalP 4.1 Server program (http://www.cbs.dtu.dk/services/SignalP/). Glycosylation site were elucidated using the Web-based-tools (http://www.cbs.dtu.dk/services/NetNGlyc/). The 3D structure of *CqTRP1* protein was predicted by SWISS-MODEL server (http://swissmodel.expasy.org/).

2.6. Tissue distribution profile of CqTRP1 mRNA

Hemocytes, Hpt, gonad, nerve, stomach, heart, gill, hepatopancreas, muscle, eyestalk, intestine and epithelium were dissected from three random individuals free of WSSV for total RNA extraction, respectively. Total RNA was extracted as described above. The mRNA expressions of *CqTRP1* in different tissues were determined by quantitative real-time RT-PCR (RT-qPCR). The PCR solution was performed in a total volume of 20 μL, containing 10 μL of 2 × SYBR Green Mix (Roche, USA), 1 μL each of primers (10 mM), 1.0 μL of

Table 1
Primer sequences used in the experiments.

Primers	Sequences (5′-3′)
CqTRP1-5′RACE	CCTCAATACCCTCACGGAGAGCCTG
CqTRP1-3′RACE	GATGCGGAAAAGGGTCAACAGGCTC
UPM	CTAATACGACTCACTATAGGGC
CqTRP1-ORF-F	ATGAGCACCGAATCCTCACTCAA
CqTRP1-ORF-R	TTAGGCTTTCTTCTCCTGCTCAATC
dsCqTRP1-F	TAATACGACTCACTATAGGGCGTATTCATTTCTCACATTTTATTG
dsCqTRP1-R	TAATACGACTCACTATAGGGTCAAAGGGAGCGGGGAGAAGGATA
dsGFP-F	TAATACGACTCACTATAGGGCGACGTAAACGGCCACAAGT
dsGFP-R	TAATACGACTCACTATAGGGTTCTTGTACAGCTCGTCCATGC
CqTRP1-qPCR-F	ATGAGCACCGAATCCTCACTCAA
CqTRP1-qPCR-R	TTAGGCTTTCTTCTCCTGCTCAATC
IE1- qPCR-F	CTGGCACAACAACAGACCCTACC
IE1- qPCR-R	GGCTAGCGAAGTAAAATATCCCCC
16S-F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA

cDNA and 8 μL of sterile water. The specific primers of *CqTRP1* and 16SRNA were designed for qRT-PCR (Table 1). The 16S ribosomal gene of *C. quadr*icarinatus (Genbank: AF135975.1) was used as a control. The qRT-PCR program used was as follows: 50 °C, 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All results were normalized to internal standard (16S RNA was used as internal standard in this study). Data of CqTRP1 expression levels were calculated using the $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT = \Delta CT$ CqTRP1-ΔCT16SRNA) method and were presented as the mean ± SD from three independent experiments (Livak and Schmittgen, 2001). The data were statistically analyzed by one-way analysis of variance.

2.7. RNAi and WSSV infection assays in Hpt cells

Double-stranded RNA (dsRNA) was synthesized to silence *CqTRP1* for its function study. The specific primers (Table 1) for *CqTRP1* and Green fluorescent protein (*GFP*) were designed and incorporated with T7 promoter at 5′ ends for RNA interference assay. The dsRNA of GFP was used as a control. In order to prepare template for dsRNA synthesis, the PCR products were amplified and purified by gel extraction. Finally, the dsRNA was synthesized by the MegaScript kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Hpt cell of *C. quadricarinatus* were prepared as described above, and cultured in 24-well plates to study WSSV replication in different time points. The Hpt cells received dsRNA treatment as follows: 1 μg of dsRNA/well was mixed with 17 μL of DEPC water and with 2 μL of Cellfectin II Reagent (Life Techologies), incubated for 10 min at room temperature, followed by mixture with 80 μL of L-15 medium, and added into the 1-day-old Hpt cell cultures. The cells were then incubated for 24 h at 20 °C. Then, the dsRNA transfection was repeated as described above. The Hpt cells were infected by WSSV after 36 h of incubation at 20 °C. The cells were collected with lysis at the time of 3 h, 6 h and 12 h after WSSV infection (MOI = 1) followed by RNA extraction, respectively. The total RNA was extracted using the GenElute™ Mammalian Total RNA miniprep kit (Sigma) according to the protocol and cDNA synthesis was described in section 2.3 using the PrimeScript™ RT Reagent Kit (TaKaRa). The RNAi efficiency of *CqTRP1* was evaluated by RT-qPCR as mentioned above. Then, the detection and comparative quantification of WSSV replication in Hpt cell cultures was done in the absence of *CqTRP1*. The expression of the immediate early gene (IE1) of WSSV was analyzed during WSSV infection. The primers used were in Table 1. The 16S RNA of crayfish was used as control.

2.8. Expression and purification of recombinant CqTRP1 from the yeast Pichia pastoris

To further explore the functions of CqTRP1, a *P. pastoris* yeast expression system was used in the preparation of recombinant CqTRP1 protein. A polyhistidine (6 × his) tag sequence was introduced into the *CqTRP1* gene sequence for detection and purification of a recombinant CqTRP1 protein. The specific primers were designed for PCR contained the underlined *Eco*R I and *Xba I* restriction sites, respectively. PCR was carried out, and the products were gel-purified. Then, the resulting PCR product and the expression vector pPICZaA (Invitrogen) were digested with the enzymes *Eco*R I and *Xba I* (TaKaRa), gel-purified, and ligated together. We used the enzyme *Bam*H I to implement the linearization of the vector before transformation into the host *P. pastoris* GS115 cells via electroporation. A single yeast colony was selected from a yeast extract peptone dextrose medium incubated for 68 h at 28 °C, and was grown with 50 mL of buffered glycerol-complex medium [composed of 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34% Yeast Nitrogen Base(YNB), 1% glycerol, 0.4 μg/mL biotin, buffered with double distilled water, BMGY] in a 250 mL conical flask. The culture was grown at 28 °C in an incubator shaker at 230 rpm for about 24 h. To induce expression in 300 mL of buffered methanol-complex medium [composed of 1% (w/v) yeast extract, 2% (w/v) peptone, 1.34 %YNB, 1% methanol, 0.4 μg/mL biotin, buffered with double distilled water, BMMY] in a 2 L flask and grown at 28 °C with shaking. The samples were withdrawn at the time of 12 h and 24 h to carry out a protein content detection assay. Fractions were analyzed by 12% SDS—PAGE.

A Ni-NTA agarose column was used to purify rCqTRP1 with a 6 × His Tag as mentioned above (Zhang et al., 2013). The inducible expression supernatant was separated from the culture media by dialyse in a dialysis bag with 50 mM PBS buffer (PH = 7.5) for 36 h, then collected with a Ni-NTA agarose column after filtration through a 0.45 μM filter membrane. The rCqTRP1 protein was eluted using buffer containing 300 mM imidazole on an AKT purifier UPC 100 (GE, USA). The concentration of rCqTRP1 was estimated by a pierce™ BCA protein assay kit (Thermo Scientific, USA), and the purification quality was analyzed by 15% SDS-PAGE. Moreover, the corresponding SDS-PAGE protein bands were identified using MALDI-TOF/TOF mass spectrometry.

2.9. Recombinant CqTRP1 antiviral assay in crayfish Hpt cells

The antiviral activity of rCqTRP1 against WSSV replication in crayfish Hpt cells was determined by RT-qPCR to analyze the

expression of the immediate early gene IE1 of WSSV. Hpt cells were seeded into 96-well and 24-well culture plates and cultured in 100 μL and 500 μL of L15. Briefly, for 24-well culture plates, 4 μL of PULSin™, 100 μL 20 mM Hepes and 1 μg rCqTRP1 were used to prepare mixture of solution. For 96-well culture plates, 1 μL PULSin™, 20 μL 20 mM Hepes and 0.3 μg of rCqTRP1 were used. The mixed solution was incubated at room temperature for 15 min, and then added into each well. After 4 h incubation, the medium was removed, and cells were washed with PBS, and 96-well culture plates was collected with cell lysis buffer 1 × SDS for Western blot (WB), 24-well culture plates was treated with WSSV (MOI = 1). Cells treated with rGFP were used as the control. The cells were collected with lysis at the time of 6 h after WSSV infection followed by RNA extraction. The total RNA was extracted using the GenElute™ Mammalian Total RNA miniprep kit (Sigma) and cDNA synthesis was described in section 2.3 using the PrimeScript™ RT Reagent Kit (TaKaRa). The expression of the immediate early gene (IE1) of WSSV was analyzed during WSSV infection. The primers used were in Table 1. The 16S RNA of red claw crayfish was used as control. The transfection efficiency of rCqTRP1 in Hpt cells was identified by WB.

3. Results and discussion

3.1. Gene cloning and characterization of CqTRP1 cDNA

A full-length cDNA of *CqTRP1* was obtained from the Hpt of red claw crayfish *C. quadricarinatus* by RACE method. The *CqTRP1* cDNA was 582 bp with an ORF of 387 bp encoding 129 amino acid residues. As shown in Fig. 1, the nucleotide contained a 92 bp of 5′-Untranslated Regions (5′-UTR) and a 103 bp of 3′-UTR with a stop codon (TAA). The ORF of *CqTRP1* encoded a polypeptide of 128 amino acids and the deduced molecular mass was 14.3 kDa with a theoretical isoelectric point of 5.3. The potential N-linked glycosylation sites in *CqTRP1* were predicted by NetNGlyc 1.0 server, and a putative N-glycosylation site was found at 58–60 amino acid sites (Fig. 1). In addition, sequence analysis by SignalP 4.1 software predicted that this gene had no signal peptide sequence, indicating that CqTRP1 did not belong to the family of secretory proteins. SMART analysis showed that *CqTRP1* protein contained three thymosin domains (thymosin β actin-binding motif), and each domain was consisted of 31–35 residues (Figs. 1 and 2c). It was reported that tetra thymosin β from *C. elegans* bound to multiple actin monomers through different repeated domains (Van et al., 2004). It has been reported that the thymosins from invertebrate have multiple thymosin domains, and the number of domains varies widely ranging from 2 (*Drosophila melanogaster, ciboulot* NP_726909.1) to 27 (*Hydra vulgaris*, *thypedin*, AAW82079.1) (Zhang et al., 2011). In contrast, the thymosins have only one domain in vertebrates (Van et al., 1996), which is a thymosin β actin-binding motif bound to actin monomers. Thus, the difference in the number of thymosin domains suggested that the thymosins might function more complicatedly in invertebrates than that of vertebrates.

In addition, sequence comparison of *CqTRP1* with the Genbank sequence datebases showed that *CqTRP1* had a high identification with β-thymosins from different invertebrates such as *P. leniusculus* (AFV39708.1), *P. clarkii* (ADY80039.1), *P. monodon* (AIW64741.1), *E. sinensis* (ACP19740.1) and *Nilaparvata lugens* (XP_022206319.1), with the identities ranging from 93% to 99%. Multiple sequence alignment of C*qTRP1* indicated that the highly conserved β-thymosin actin-binding motif in other species was also present in red claw crayfish *C. quadricarinatus* (Fig. 2a). The *CqTRP1* of *C. quadricarinatus* showed closer evolutionary relationships with *EsTRP1* of *E. sinensis.* These data suggested that *CqTRP1* was a member of the β-thymosin family which might have the similar biological function with β-thymosin of vertebrates.

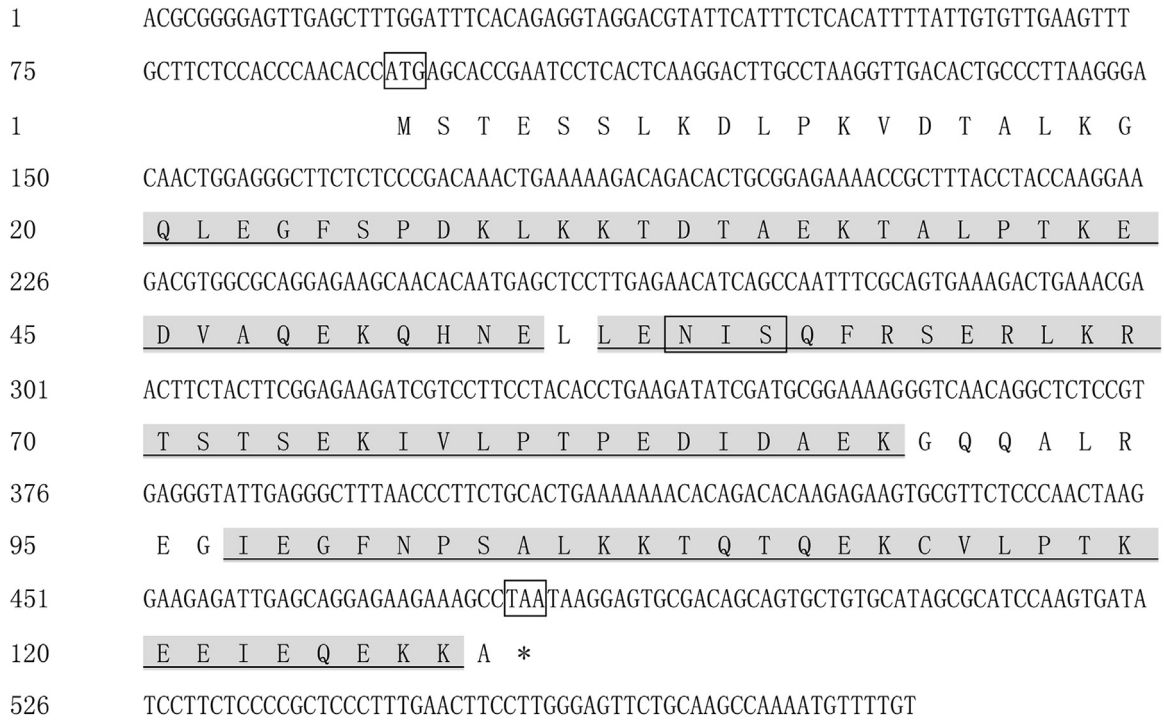

Fig. 1. Nucleotide and deduced amino acid sequences of *CqTRP1* cDNA from *C. quadricarinatus*. The nucleotide sequence of *CqTRP1* contained start and stop codons. The three thymosin β actin-binding domains were underlined and shadowed. The amino acid sequences in the box indicated the putative N-glycosylation sites.

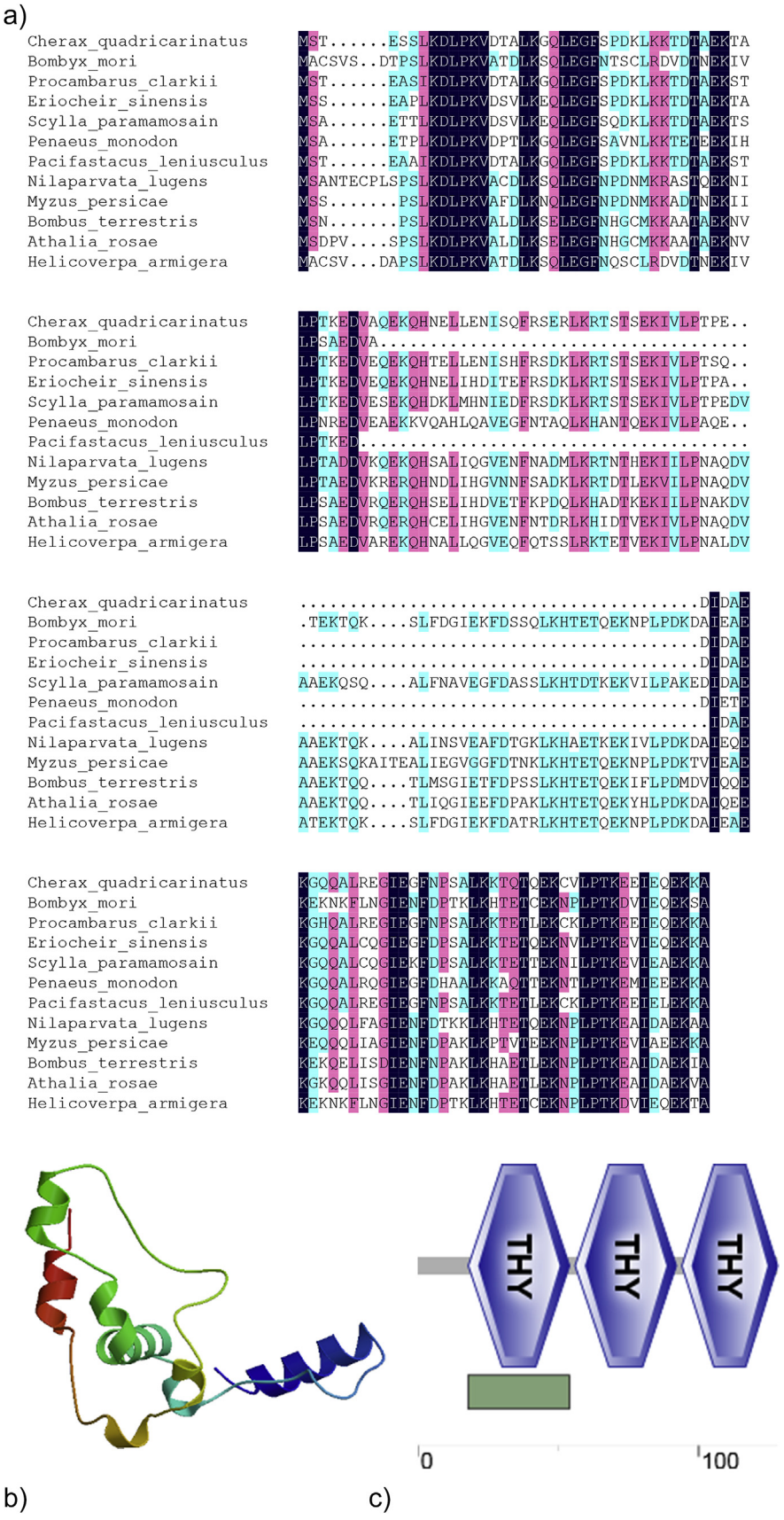

Fig. 2. Multiple alignment of *CqTRP1*.

(a) Sequence alignment of *CqTRP1* with other known thymosins: *Bombyx mori* (Q1HPK4); *Procambarus clarkii* (ADY80039.1); *Eriocheir sinensis* (ACP19740.1); *Scylla paramamosain* (ACY66642.1); *Penaeus monodon* (AIW64741.1); *Pacifastacus leniusculus* (AFV39707.1); *Nilaparvata lugens* (XP_022206319.1); *Myzus persicae* (XP_022173465.1); *Bombus terrestris* (XP_003399117.1); *Athalia rosae* (XP_012256154.1) and *Helicoverpa armigera* (XP_021184280.1); The black boxes enclose the conserved thymosin amino acid residues.

(b)The spatial structure model of *CqTRP1*.

(c) Predicted protein domain structure of *CqTRP1*. *CqTRP1* contained three thymosin β actin-binding motifs.

3.2. Tissue distribution of CqTRP1 at transcriptional level in red claw crayfish

To analyze the expression profiles of *CqTRP1* in different tissues, we investigated the transcription levels of *CqTRP1* by RT-qPCR. The results showed that the expression levels of *CqTRP1* was different in the selected tissues (Fig. 3). The mRNA transcripts of *CqTRP1* were highly expressed in hemocytes, followed by Hpt, but were less expressed in epithelium, intestine and eyestalk. Previous studies have shown that the *EsTRP1* transcript was highly expressed in hemocytes in Chinese mitten crab *E. sinensis* (Gai et al., 2009). The transcriptional level of *PcThy-4* was with highest expression in heart, hemocytes and gills in red swamp crayfish *P. clarkii* (Shi et al., 2015). In the freshwater crayfish *P. leniusculus*, the transcripts of Pl-β-thymosin1 and Pl-β-thymosin2 were highly expressed in the Hpt, brain and hemocytes (Saelee et al., 2013). In our present study, the *CqTRP1* was mainly distributed in hemocytes. It is well-known that hemocytes play a key role in the integration of extracellular signals in invertebrates. Furthermore, hemocytes participate in recognition, phagocytosis, melanization of pathogens and cytotoxicity. It has been reported that β-thymosin is one of the most abundant intracellular protein families and is involved in cell migration, attachment and spreading of cancer (Cha et al., 2003; Kim et al., 2009; Larsson and Holck, 2007; Tang et al., 2011). Taken together, the expression profile of the β-thymosin gene indicated that *CqTRP1* might participate in host immune response in hemocyte in a crustacean *C. quadricarinatus*.

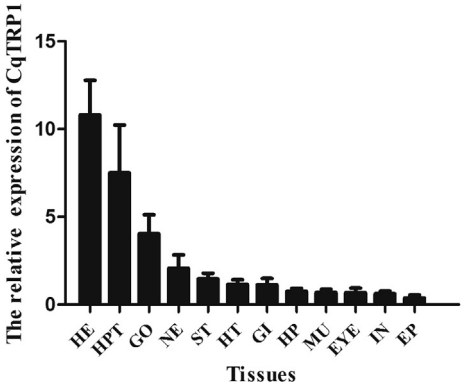

Fig. 3. The distribution of *CqTRP1* mRNA in different tissues from *C. quadricarinatus*. Significant differences ($^{*}p < 0.05$) of *CqTRP1* expression were indicated with different letters between different tissues. HE: haemocyte; Hpt: hematopoietic tissue; GO: gonad; NE: nerve; ST: stomach; HT: heart; GI: gill; HP: hepatopancreas; MU: muscle; EYE: eyestalk; IN: intestine and EP: epithelial tissue. Vertical bars represent the mean ± SD ($n = 3$).

3.3. Enhanced WSSV replication by gene silencing of CqTRP1 in Hpt cells from red claw crayfish

Recently, it was shown that β-thymosin had a role in defense against WSSV infection in red swamp crayfish *P. clarkii*, which affected crayfish survival rate by inhibiting the WSSV replication (Shi et al., 2015). Previously, we found that *CqTRP1* was significantly increased after WSSV challenge in red claw crayfish Hpt cells (Liu et al., 2011). In order to elucidate whether *CqTRP1* participated in WSSV infection in red claw crayfish, a RNAi assay on endogenous *CqTRP1* was carried out in Hpt cells followed by WSSV infection. The gene knockdown efficiency of *CqTRP1* was decreased more than 93% in silenced cells in comparision to the controls at each time point (Fig. 4a). However, no significant difference of WSSV entry or "uptake" was found between CqTRP1 silenced Hpt cells and GFP dsRNA treated control cells (data not shown). To determine the role of *CqTRP1* in WSSV infection, we examined the replication of WSSV after gene silencing of the *CqTRP1* in Hpt cells. Then, the relative expression of an immediate early gene (IE1) of WSSV, an indication of the replicated WSSV at the viral infection stage with 3, 6 and 12 h post WSSV infection, was significantly increased, which demonstrated that the replication of WSSV was increased in the Hpt cells of the *CqTRP1* silenced cells (Fig. 4b). In vertebrate, thymosin is a product of thymic epithelial cells and both the humoral and cellular responses are significantly regulated by thymosin (Goldstein et al., 1966; Mandi and Glant, 1973). As a major member of the thymosin β family, thymosin β4 was an important antimicrobial peptide and anti-inflammatory agent in vertebrate. While the thymosin β4 of human was responsive to rhinovirus infection (Forger and Cerny, 1976). Also, a preventive role of Tβ4 in septic hypercirculation highlighted that Tβ4 acted as a potential therapeutic target in severe sepsis in C57BL/6 mice (Bongiovanni et al., 2015). Moreover, thymosin β4 was involved in the regulation of chronic inflammation and fibrosis, which played a defensive role in the disease progression of chronic hepatitis B (CHB) combined nonalcoholic fatty liver disease (NAFLD) patients (Liang et al., 2016). The thymosin β of *Cyprinus carpio* was shown to enhance the immune response during spring viraemia of carp virus (SVCV) infection and

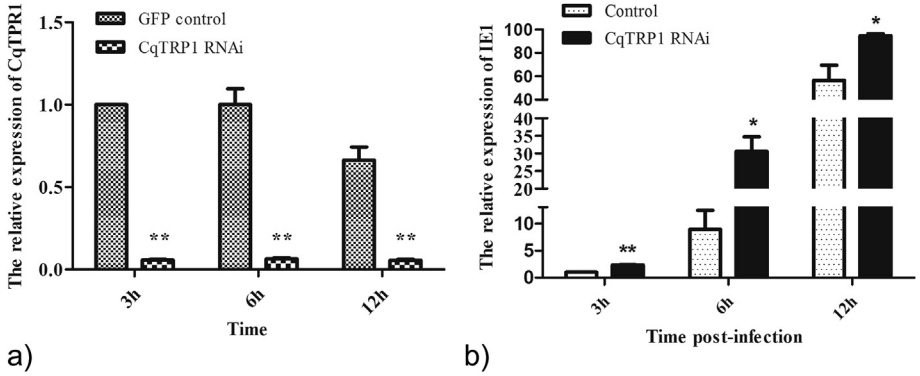

Fig. 4. Increased WSSV replication by gene silencing of *CqTRP1* in red claw crayfish Hpt cells.
(a) RNAi efficiency of *CqTRP1* was detected by qRT-PCR before WSSV infection. The results showed that *CqTRP1* gene expression was significantly reduced at 3, 6 and12 h by silencing of *CqTRP1* with dsRNA ($^{**}p < 0.01$) . The GFP dsRNA treatment was used as a control. Values were shown as mean ± S.E ($n = 3$).
(b) WSSV IE1 expression in *CqTRP1* silenced Hpt cells. The mRNA expression level of IE1 was measured by RT-qPCR in *CqTRP1* silenced Hpt cells followed by WSSV challenge at different time intervals. Values were shown as mean ± S.E ($n = 3$). Significant differences in IE1 expression were indicated by different number of asterisk between ds*CqTRP1* and ds*GFP* group ($^{**}p < 0.01$, $^{*}p < 0.05$).

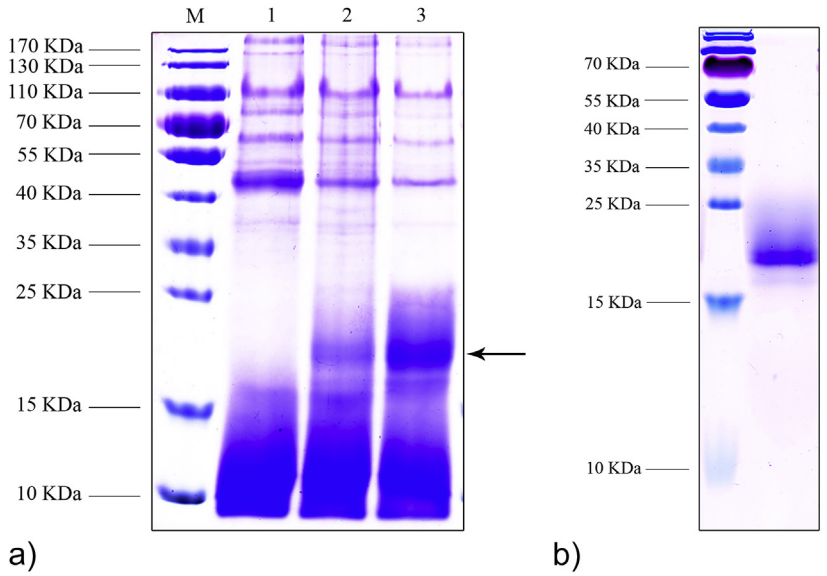

Fig. 5. Purification of rCqTRP1 in *P. pastoris*.
(a) SDS-PAGE analysis of crude product from *P. pastoris*. Lane M: marker proteins; lane 1: protein components of cultured medium from the pPICZαA/CqTRP1 recombinant clone before induction by methanol; lane 2, 3: The expression of rCqTRP1 protein induced by methanol at 12 and 24 h, respectively.
(b) SDS-PAGE analysis of rCqTRP1 purification. The rCqTRP1 was purified by affinity chromatography using Ni−NTA and visualized on 15% of SDS−PAGE gel.

modulate the development of T lymphocytes in teleost (Xiao et al., 2015). Besides, some β-thymosins have been identified to inhibit WSSV infection in *E. sinensis, P. clarkii* and *Marsupenaeus japonicus* (Gai et al., 2009; Ma et al., 2016; Shi et al., 2015). However, there is little information about the antiviral mechanism of crustacean β-thymosins. For instance, it was only reported that β-thymosins could promote hemocyte phagocytosis of WSSV and inhibition of WSSV replication in red swamp crayfish *P. clarkii* (Shi et al., 2015). In the larvae of *Helicoverpa armigera*, two thymosin isoforms HaTHY1 and HaTHY2 were induced by bacterial and viral challenge, indicating their roles in insect immunity (Zhang et al., 2011). These data together indicated that β-thymosin is likely to possess an important immune function in invertebrates which still needs furthermore investigations.

3.4. Expression, purification and antiviral activity of rCqTRP1

In order to find out whether extra CqTRP1 protein could inhibit WSSV replication in Hpt cells, a mature CqTRP1 protein was recombinantly expressed with the pPICZaA vector in the yeast *P. pastoris* and further purified via single-step purification using a Ni-NTA affinity column. As shown in Fig. 5a, an approximately 18 kDa glycosylated protein was induced by methanol and secreted into the culture medium detected by SDS-PAGE. The amount of rCqTRP1 at 24 h (Fig. 5a, lane 3) was more than that of 12 h (Fig. 5a, lane2) after induction by methanol. The purified rCqTRP1 was approximately 18 kDa in size (Fig. 5b). Actually, the deduced molecular mass of CqTRP1 was 14.3 kDa. We speculated that putative glycosylation might occur in CqTRP1 during recombinant protein expression in the eukaryotic expression system. The rCqTRP1 protein bands were confirmed by MALDI-TOF/TOF mass spectrometry analysis, which demonstrated that several peptide fragments corresponded to the deduced protein sequences of *CqTRP1* (data not shown). To further investigate if the CqTRP1 could interfere with the WSSV replication, the rCqTRP1 was added into Hpt cells followed by infection with WSSV. The result showed that the extra rCqTRP1 in Hpt cells was detected by Western blotting, indicating a successful protein introduction if compared to that of rGFP group

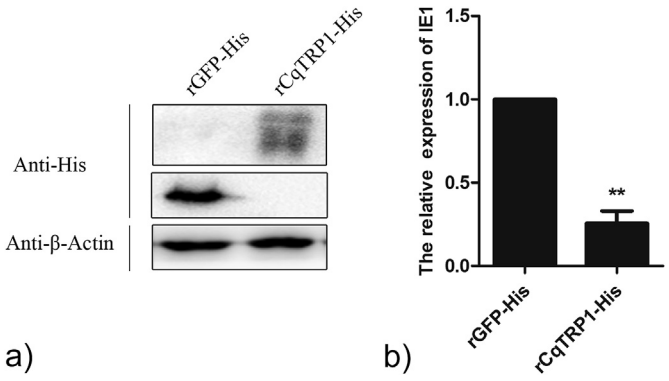

Fig. 6. Decreased WSSV replication by extra rCqTRP1 in red claw crayfish Hpt cells.
(a) The anti-His antibody was used to detect rCqTRP1 and rGFP after overloading of recombinant protein into Hpt cells.
(b) The transcription of IE1 was inhibited after the introduction of rCqTRP1 into HPT cells. Hpt cells were introduced with extra-rCqTRP1 and extra-rGFP followed by WSSV challenge for 6 h. The mRNA expression of IE1 was examined by RT-qPCR. Values were shown as mean ± S.E ($n = 3$) ($^{**}p < 0.01$).

(Fig. 6a). Furthermore, the gene expression of WSSV IE1 was markedly decreased in Hpt cells compared to that of control group (Fig. 6b). Therefore, rCqTRP1 had anti-WSSV property in red claw crayfish, which could be employed as a putative anti-viral factor in crustacean aquaculture.

4. Conclusions

In conclusion, a *CqTRP1* gene was characterized from red claw crayfish *C. quadricarinatus*. Functional study showed that *CqTRP1* played a key role in anti-WSSV response in a crustacean. Hence, these data provides information for future studies on antiviral mechanism and further viral disease control in crustaceans.

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