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Identification of a *CqCaspase* gene with antiviral activity from red claw crayfish *Cherax quadricarinatus*

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ABSTRACT

Caspase, an aspartate specific proteinase mediating apoptosis, plays a key role in immune response. In our previous study, the expression of a caspase gene was up-regulated in a transcriptome library from the haematopoietic tissue (Hpt) cells of red claw crayfish *Cherax quadricarinatus* post white spot syndrome virus (WSSV) infection. To further reveal the effect of caspase on WSSV infection, we cloned this caspase gene (denominated as *CqCaspase*) with an open reading frame of 1062 bp, which encoded 353 amino acids with a caspase domain (CASc) containing a p20 subunit and a p10 subunit. Tissue distribution analysis indicated that the mRNA transcript of *CqCaspase* was widely expressed in all tested tissues with the highest expression in Hpt, while the lowest expression in muscle. To further explore the effect of *CqCaspase* on WSSV replication, recombinant protein of *CqCaspase* (rCqCaspase) was delivered into Hpt cells followed by WSSV infection, which resulted in a significantly decreased expression of both an immediate early gene IE1 and a late envelope protein gene VP28 of WSSV, suggesting that *CqCaspase*, possibly by the enhanced apoptotic activity, had a strong negative effect on the WSSV replication. These data together indicated that *CqCaspase* was likely to play a vital role in immune defense against WSSV infection in a crustacean *C. quadricarinatus*, which shed a new light on the mechanism study of WSSV infection in crustaceans.

1. Introduction

Apoptosis is a form of programmed cell death (PCD) (Samali et al., 1999), which was firstly proposed by Kerr in 1972 (Kerr et al., 1972). The process of apoptosis is mainly mediated by caspase (cysteinyl aspartates specific proteinase), which cleaves the target proteins at aspartate residues relying on its active site (Alnemri et al., 1996). Caspase was first identified from *Caenorhabditis elegans* called *ced-3* (cell-death abnormality-3) in 1993 (Yuan et al., 1993). Up to now, at least 15 kinds of distinct caspases have been identified from mammals. According to biological activity, caspases could be divided into pro-inflammatory caspase (caspase-1, 4, 5, 12 in humans and caspase-1, 11, 12 in mice) and pro-apoptotic caspase which are sub-classified into the initiator caspases (caspase-2, 8, 9, 10) and effector caspases (caspase-3, 6, 7) (Hakem et al., 1998; Lawen, 2003; Takle and Andersen, 2007). Similarly, caspases have been identified from crustaceans, like *Penaeus merguiensis*, *Macrobrachium rosenbergii*, *Penaeus monodon* (Arockiaraj et al., 2012; Leu et al., 2008; Phongdara et al., 2006). and *Eriocheir sinensis* (Jin et al., 2011). In most cases, caspases possess the caspase domain (CASc) containing a large subunit (p20) and a small subunit (p10) at C-terminal, and a prodomain at N- terminal (Fan et al., 2005). It has been reported that apoptosis plays crucial roles in many physiological processes, including embryonic development, organismal aging and homeostasis maintenance (Huang et al., 2010). Importantly, apoptosis can also control the microbe infection by eliminating the harmful, dangerous, damaged or unnecessary cells. In mammals, apoptosis can be divided into the intrinsic and extrinsic apoptotic pathways. Nonetheless, apoptotic pathway is not clear in crustaceans. And the previous studies of caspases mainly focus on the gene cloning and expression profiles in crustacean, but the protein function of caspase, especially, the direct effect on white spot syndrome virus (WSSV) infection, is not well-defined. As we know, WSSV is a lethal pathogen for crustacean aquaculture, particular for the shrimp and crayfish aquaculture, and causes a large economic loss (Escobedo-Bonilla et al.,

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2008; Lightner, 2011). Therefore, it is necessary to find the efficient method to control WSSV disease. As is well-known, apoptosis mediated by caspase is one kind of the key innate immunity against viral infection not only in vertebrates but also in crustaceans. For instance, the loss-of-function of *Pjcaspase* inhibited the apoptosis induced by WSSV in *Marsupenaeus japonicas*. Meanwhile, the viral copies was clearly increased after gene silencing of *Pjcaspase*, which indicated that apoptosis played a key role in antiviral process of shrimp (Wang et al., 2008). And it was reported that caspase was likely to be responsive to WSSV infection in shrimp. For example, *Pmcaspase* exhibited the caspase-3 activity *in vitro* and the gene expression was increased post WSSV challenge in *P. monodon* (Wongprasert et al., 2007). Besides, the gene expression of *Lvcaspase2-5* was up-regulated in haemocyte after WSSV infection, and the expression of a viral late gene VP28 of WSSV was increased when *Lvcaspases* were knocked down *in vivo* post WSSV infection in *Litopenaeus vannamei*, which indicated that *Lvcaspases* played a key role in defence against WSSV (Wang et al., 2013a). Based on the study in transcript expression of caspase, the interaction between WSSV protein and caspase have also been investigated. For example, WSSV protein rWSSV134 could interact with the p20 subunit of *PmCasp* and inhibit its activity in a dose-dependent manner, implying that WSSV134 might benefit the viral blocking on apoptosis in shrimp cells by suppressing *PmCasp* activity (Bowornsakulwong et al., 2017; Lertwimol et al., 2014). But the molecular details in regulation are not clear.

Previously, we found that the gene expression of *CqCaspase* (denominated as *CqCaspase*) was up-regulated in haematopoietic tissue (Hpt) cells from red claw crayfish *Cherax quadricarinatus* after WSSV infection from a transcriptome library in our lab (unpublished data), indicating that *CqCaspase* might be involved in the host response to WSSV infection while needing further investigation. In the present study, we identified a *CqCaspase* gene from *C. quadricarinatus* and the expression profile was determined. Furthermore, the effect on WSSV replication by *CqCaspase* was investigated, which indicated that *CqCaspase* exhibited a strong inhibition on WSSV replication.

2. Materials and methods

2.1. Experimental animals and samples preparation

Healthy red claw crayfish *C. quadricarinatus* were purchased from Yuansentai Technology Co. Ltd, Zhangzhou, Fujian Province, China. Red claw crayfish with body weight of 48 ± 2 g and 70 ± 2 g were used for tissues collection and Hpt cells cultures, respectively. All the crayfish were acclimated in aerated freshwater tanks at 26 °C for at least one week before experiments.

Haemocyte was collected with a sterile syringe with equal volume of anticoagulation and centrifuged for 10 min with 1000 × *g* at 4 °C. Other tissues, including stomach, gonad, muscle, nerve, intestine, heart, Hpt, hepatopancreas, gill, epithelium and eyestalk were collected from three random crayfish for RNA extraction. Hpt cell cultures were prepared in a 24-well plate and a 96-well plate, respectively, as previously described (Liu et al., 2011; Söderhäll et al., 2003).

2.2. RNA extraction and cDNA synthesis

The total RNA of each tissue was isolated with Trizol regent (Roche, Mannheim, Germany) according to the manufacturer's protocols. RNase-free DNAase I was used to eliminate the genomic DNA in total RNA. The RNA concentration and quality were assessed by Nanodrop 2000 (Thermo Scientific, USA) followed by cDNA synthesis with PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacture's instruction.

2.3. Gene cloning of the full-length cDNA sequence of CqCaspase

The partial open reading frame (ORF) sequence of *CqCaspase* was isolated from a transcriptome library of Hpt cells post WSSV infection in our lab (unpublished data). To clone the full-length ORF sequence of *CqCaspase*, the primers of *CqCaspase* (CqCaspase-F and CqCaspase-R) were designed using Primer 5.0. The sequences of primers were shown in Table 1. The PCR reaction conditions were as follows: 5 min at 94 °C; 30 cycles of 98 °C for 10 s, 65 °C for 15 s and 72 °C for 20 s; and 72 °C for 10 min. The PCR production was gel-purified with 1.2% agarose gel using a Gel Extraction Kit (Sangon Bioteach, Shanghai, China) and ligated into pMD18-T vector (TaKaRa). Then the vector was transformed into *Escherichia coli* DH5α cells and the positive clones containing the inserts of an expected size were sequenced at Xiamen Borui Biotech Company, China.

Table 1
The primers sequences used in this study.

Primers	Sequences
CqCaspase-F	CCG**GAATTC**ATGATTCACATGTGTGATAGTTTGA
CqCaspase-R	ATAAGAAT**GCGGCCGC**TTACTGTTTCTTTTTACCCACTGGA
qCqCaspase-F	AAGCAAAGATGAAGGTTCAGTGTT
qCqCaspase-R	TCATAGGAACATGCTTGTTTGG
16S-F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC
VP28-F	AAACCTCCGCATTCCTGT
VP28-R	GTGCCAACTTCATCCTCATC

2.4. Sequence analysis of CqCaspase

The amino acid sequence of *CqCaspase* was deduced with ExPASy translate tool (http://web.expasy.org/translate/). The homologous conserved domains were identified by SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de) and ExPASy prosite (http://prosite.expasy.org/prosite.html). The 3D structure of *CqCaspase* protein was constructed using SWISS-MODEL server. And the multiple sequences alignment of domain in *CqCaspase* and other caspases were performed using DNAMAN 6.0.3 program. A phylogenetic tree was constructed by the maximum likelihood algorithm using the Mega 6.0 software.

2.5. Tissues distribution analysis of CqCaspase gene in red claw crayfish

The mRNA expression of *CqCaspase* in different tissues from *C. quadricarinatus* were detected by quantitative real-time PCR (qRT-PCR) using an ABI PCR machine (Applied Biosystems 7500, UK). The 16S rRNA (Genbank ID: AF135975.1) in the red claw crayfish was used as an internal control. The primers for detection of *CqCaspase* (qCqCaspase-F and qCaspase-R) and 16S rRNA (16S-F and 16S-R) were shown in Table 1. The reaction of qRT-PCR was comprised of 10 μL of SYBR Green Master (2×) (Roche, USA), 2 μL of primer pairs (10 μM), 1 μL of cDNA for target gene or 1 μL of 50 times diluted cDNA for 16S rRNA, and 7 μL of sterile water. Thermal cycling conditions for the qRT-PCR was performed with 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curve analysis was used to confirm the specificity of qRT-PCR amplification. The relative transcript expression levels were calculated by using the $2^{-\triangle\triangle Ct}$ method. This experiment was performed for three times.

2.6. Recombinant expression and purification of recombinant CqCaspase protein

The coding region of *Cqcasepase* was amplified from pMD18-T/CqCaspase recombinant vector with a pair of specific primers which contained *Eco*R I and *Not* I endonuclease sites (Table 1). After gel-purification, the target PCR production and expression vector pET28a

were digested using endonuclease *Eco*R I and *Not* I. The recombinant vector pET28a-CqCaspase was ligated and transformed into *E.coli* BL21 cells to express the recombinant CqCaspase protein (rCqCaspase) induced by 0.1 mM isopropylthiogalactoside (IPTG) for 20 h at 16 °C. The *E.coli* cells were collected with centrifugation (8000 × *g* for 10 min), then the pellet was resuspended in PBS. Following ultrasonic processing of the cells, the supernatant was reserved by centrifugation (12,000 × *g* for 30 min). The supernatant was incubated with NiResin FF beads for 2 h at 4 °C. After rising with PBS containing 20 mM imidazole, the protein was eluted with PBS containing 100 mM imidazole and dialysed in PBS for 48 h. The purified protein was analyzed by SDS-PAGE.

2.7. Effect on WSSV replication by delivery of recombinant CqCaspase protein into Hpt cells

To investigate the effect on WSSV infection by *CqCaspase*, the delivery of rCqCaspase protein into Hpt cells was performed. Briefly, to determine whether protein was delivered into Hpt cells successfully, the cells were cultured in 96-well plates. And 300 ng of rCqCaspase plus 1 μL of PULSin (PolyPlus transfection, French) were mixed in 20 mM HEPES buffer with a final volume of 20 μL followed by incubation for 15 min at room temperature, then appended medium was supplied up to 50 μL followed by inoculation into the cell wells. After incubation for 4 h, the medium was removed and the cells were collected by using 1 × SDS cell lysis buffer for the detection of the protein with Western blotting. Meanwhile, cells were seeded in 24-well plates for explore the effect on WSSV infection by rCqCaspase protein. One microgram of rCqCaspase with 2 μL of PULSin was mixed in 100 μL of HEPES. After incubation for 15 min at room temperature, the mixtures were added into the cell wells and incubated for 4 h followed by WSSV infection (MOI = 1). Finally, cells were collected with RNA lysis solution at 6 h post WSSV infection for transcript determination of viral genes IE1 and VP28 with qRT-PCR. The primers for detection of IE1 and VP28 were shown in Table 1. Cells treated with recombinant GFP protein with His tag were used as the control treatment. The experiment was carried out in triplicates.

For Western blotting analysis, the cells collected with 1 × SDS lysis 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, followed by incubation with mouse anti-His antisera (1:3000) and anti-β-actin (1:3000) (TransGene Biotech, Beijing, China) for 1 h at room temperature. The membranes were then gently washed three times with TBST buffer for 15 min, and subsequently incubated with HRP-conjugated goat anti-mouse secondary antibodies (1:5000) for 1 h at room temperature. After wash for three times with TBST buffer, the bands were detected by immunoblotting.

2.8. Statistical analysis

All the data were analyzed by Student's *t*-test and presented as the mean ± SD from more than three independent assays by 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 bioinformatics analysis of CqCaspase cDNA sequence

Previously, *CqCaspase* was up-regulated in a transcriptome library post WSSV infection (unpublished data). To further elucidate how *CqCaspase* functioned during WSSV infection, we then cloned *CqCaspase* gene (Genbank ID: MH974813) followed by functional identification. As shown in Fig. 1, the ORF of *CqCaspase* was 1062 bp, which encoded 353 amino acids. The calculated protein molecular weight of *CqCaspase* was 40.4 kDa with a predicted isoelectric point of

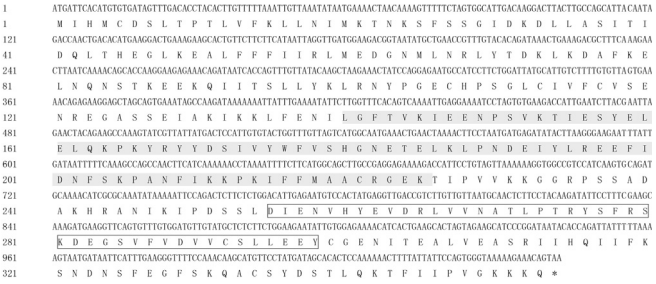

Fig. 1. Nucleotide and deduced amino acid sequences of *CqCaspase* from *C. quadricarinatus*. The p20 subunit was shown in shadow and the p10 subunit was marked in box.

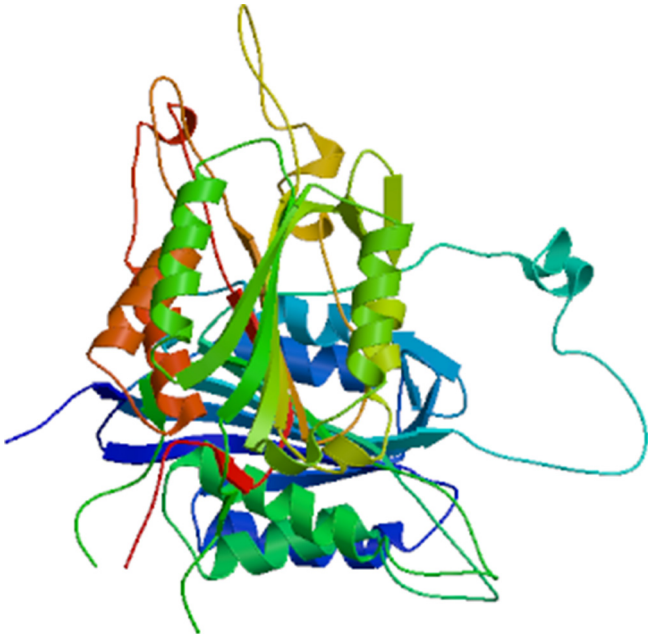

Fig. 2. The bioinformatics analysis of *CqCaspase* gene. (A) The 3D structure model of *CqCaspase* gene. The different colors represent the order of protein. The N- terminus to C- terminus was shown from blue to red. (B) The predicted protein domain structure of *CqCaspase*. The *CqCaspase* contained a CASc domain but without signal peptide. (C) The multiple sequences alignments of CASc domain of caspase genes from different species. The conserved amino acids were shown in colors. The amino acids sequences of caspase CASc domain were from crustacean, insect and mammals. The Genbank ID of amino acids sequences were shown as follows: *L. vannamei* caspase3 (AGL61582.1); *Fenneropenaeus merguiensis* caspase (AAX77407.1); *M. japonicas* caspase (ADH93986.1); *M. rosenbergii* caspase3C (AET34920.1); *P. monodon* caspase (ABI34434.1); *E.sinensis* caspase3C (AGT29868.1); *D. melanogaster* caspase-1 (AAB58237.1); *Spodoptera frugiperda* caspase-1 (AAC47442.1); *Xenopus laevis* caspase 9S (NP_001079035.1); *Homo sapiens* caspase-3 (NP_004337.2); *H. sapiens* caspase-7 (NP_001218.1); *H. sapiens* caspase-6 (NP_001217.2); *H. sapiens* caspase-9 (NP_001220.2); *H. sapiens* caspase-8 (NP_001073593.1); *H. sapiens* caspase-10 (NP_116759.2); *H. sapiens* caspase-2 (NP_116764.2). (D) The phylogenetic tree of *CqCaspase* with other caspases. The Genbank ID of sequences was shown as follows: *H. sapiens* caspase-1 (NP_001244047.1); *Mus musculus* caspase-6 (NP_033941.3); *H. sapiens* caspase-4 (NP_001216.1); *H. sapiens* caspase-5 (NP_004338.3).

6.96. SignalP analysis predicted that *CqCaspase* did not contain signal peptide. The 3D structure of *CqCaspase* was similar to the human caspase-6 (Fig. 2A). The structure analysis showed that the deduced *CqCaspase* protein sequence contained a CASc domain at C terminal region (102-348 aa), which was comprised of a large subunit p20 (140-225aa) and a small subunit p10 (255-298aa) based on the analysis by SMART and ScanProsite programs (Fig. 2B). These subunits have been

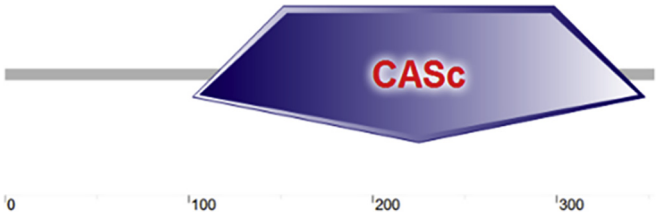

Fig. 2. (*continued*)

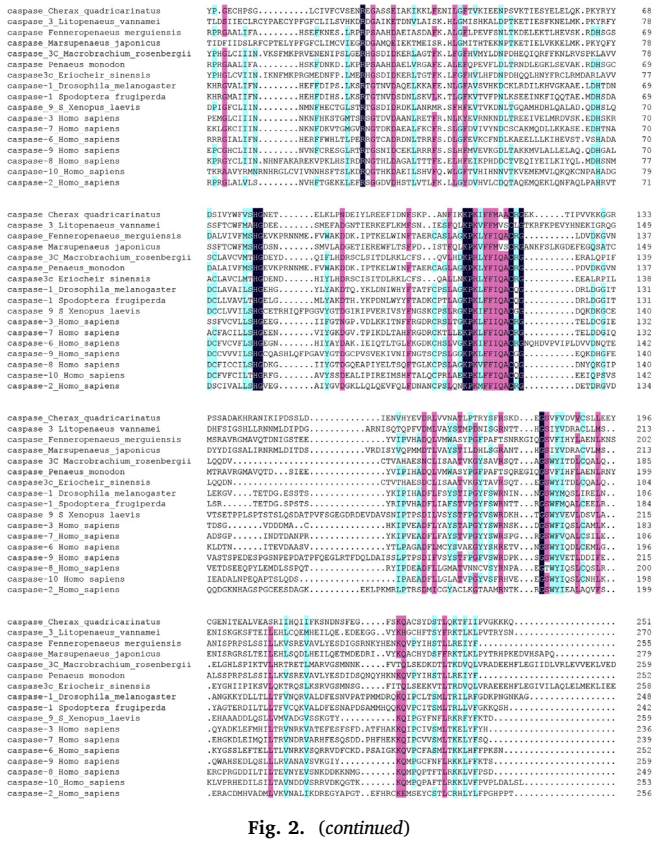

Fig. 2. (*continued*)

Fig. 2. (*continued*)

shown to play crucial roles in caspase activation and catalysis, in which caspase is activated by assembling of the p10 unit into an active hetero-tetramer proteolytic site, resulting in the proteolytic cleavage between the p20 and p10 units (Chang and Yang, 2000). Interestingly, WSSV protein WSSV134 and WSSV322 have been shown to interact with the p20 subunit of *PmCaspase* that led to the inhibition on apoptosis in *P. monodon* (Lertwimol et al., 2014). Besides, the multiple sequences alignment of caspases from insects, mammals and other crustaceans showed that the CASc domain was relatively conserved (Fig. 2C) with two active sites (His180 and Cys221), which contained one substrate pocket of 12 residues, two proteolytic cleavage sites and one dimer interface of 15 residues in *C. quadricarinatus*. Similar to other species like *E. sinensis* (Jin et al., 2011), the active site His residue was located between the serine and glycine residues of *CqCaspase*. And the other active site the Cys residue was located in a characteristic five-peptide motif AACRG at p20 subunit terminal. In most cases, caspases were secreted as inactive form and its activation was dependent on two proteolytic cleavages (Wu et al., 2014). Usually, a five-peptide motif Glu-Ala-Cys-X-Gly (QACXG, X refers to R, Q or G) was the basic feature of p20 subunit in CASc domain. However, the CASc domain of *CqCaspase* exhibited the motif of AACRG instead of QACRG. This difference also could be found in other crustaceans. For example, the motif was VSCRG in *Mjcaspasea* (Genbank ID: ADH93986.1) and VLCRG in *Escaspase* (Genbank ID: ADM45311.1). Additionally, the phylogenetic tree was constructed with caspase amino acids of insects, mammals and crustaceans by using the Mega 6.06, in which these caspases were divided into two groups. Caspase-8 and -10 from *Homo sapiens*, *Mrcaspase3c*, *Escaspase3c*, *Mjcaspases*, *Lvcaspase3* and *CqCaspase* were clustered into one group and the other caspases were separated into another group. Besides, *CqCaspase* was close to the *Mjcaspase* and *Lvcaspase3* (Fig. 2D). Caspase-8 and -10 were belonged to initiator caspase and *Lvcaspase3* was a member of initiator caspase (Wang et al., 2013a). In consideration of a 101 residues of prodomain in *CqCaspase*, we speculated that the *CqCaspase* might be an initiator caspase, which could activate the effector caspase then resulting in the cell death. Meanwhile, the BlastX analysis showed that *CqCaspase* shared 34% identity with *Mjcaspase*. Taken together, these data suggested that *CqCaspase* might have similar biological function with the caspases from other crustaceans.

3.2. Tissues distribution of CqCaspase transcript in red claw crayfish

To determine the expression profile of *CqCaspase* in different tissues from healthy *C. quadricarinatus*, the qRT-PCR was carried out. As shown in Fig. 3, *CqCaspase* was expressed in all test tissues, including Hpt tissue, haemocyte, gill, gonad, nerve, heart, epithelium, intestine, hepatopancreas, eyestalk, stomach and muscle, which was in consistent with *Escaspase-3-like* in mitten crab (Wu et al., 2014). In consideration of that caspase acts as a universal protein to induce the cell apoptosis, a wide distribution of *Cqcaspse* in different tissues indicated that apoptosis occurs in almost all the tissues or organs to eliminate the damaged or unnecessary cells in *C. quadricarinatus*. In addition, *CqCaspase* was highly expressed in Hpt tissue, haemocyte and gill, while lowest expression was detected in muscle. And the similar result could be observed for *Lvcaspase3* from *L. vannamei*, in which *Lvcaspase3* had the highest expression in haemocyte (Wang et al., 2013a). It is well known that crustacean is lacking of adaptive immunity and haemocyte plays a critical role in innate immune response against pathogens infection, including immune recognition, release of antimicrobial substances, phagocytosis, encapsulation and cytotoxicity (Johansson et al., 2000). And WSSV infection could induce apoptosis of haemocyte in *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2006). Besides, the mRNA expression of *Lvcaspases* in haemocytes of *L. vannamei* was significantly up-regulated after WSSV infection (Wang et al., 2013a). Hence, the higher expression of *CqCaspase* in haemocyte implied that *CqCaspase* might participate in the anti-WSSV response. Additionally, WSSV could

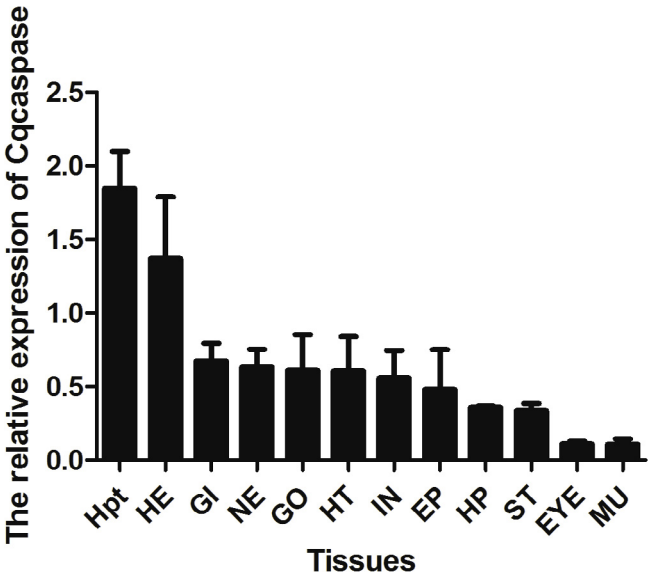

Fig. 3. The gene expression profile of *CqCaspase* in various tissues from *C. quadricarinatus*. The transcript of *CqCaspase* was detected by qRT-PCR. The 16S rRNA was taken as an internal control. Hpt: hematopoietic tissue; HE: haemocyte; GI: gill; GO: gonad; NE: nerve; HT: heart; EP: epithelium; IN: intestine; HP: hepatopancreas; EYE: eyestalk; ST: stomach; MU: muscle.

propagate successfully in Hpt tissue or cultured cells (Wu et al., 2015). Taken together, the abundant expression of *CqCaspase* in Hpt and haemocyte indicated that *CqCaspase* might play a crucial role in immune defense against WSSV infection in *C. quadricarinatus* but the mechanism still needs furthermore investigations.

3.3. The recombinant expression and purification of recombinant CqCaspase protein

To explore the biological function of *CqCaspase*, recombinant *CqCaspase* protein (rCqCaspase) with His-Tag was expressed in *E.coli* (BL21:DE3) and further purified via Ni Resin FF beads. As shown in Fig. 4, rCqCaspase protein was approximately 41 kDa, which was in consistent with the prediction of the protein molecular weight, and further confirmed by MALDI-TOF/TOF mass spectrometry analysis (data not shown). Besides, to obtain the pure rCqCaspase protein, the affinity chromatography with Ni Resin FF beads was used for protein purification. The purity of rCqcaspse was reached to 90% (Fig. 4) which was suitable for further protein functional study.

3.4. Inhibition on WSSV replication by delivery of rCqCaspase protein into Hpt cells

As mentioned above, *CqCaspase* was highly expressed in some immune-related tissues like Hpt and haemocyte in red claw crayfish. Besides, our previous study showed that the *CqCaspase* expression was up-regulated after WSSV infection in red claw crayfish. Therefore, we speculated that rCqCaspase might have certain effect on WSSV infection. To test this hypothesis, the rCqCaspase protein was delivered into Hpt cells by chemical kit followed by WSSV infection at 4 h post protein delivery, and the delivery of recombinant GFP protein with His tag was used as the control. As shown in Fig. 5A, both the rCqCaspase and rGFP protein were detected in Hpt cells with Western blotting by using anti-His antibody, which suggested that rCqCaspase was successfully delivered into Hpt cells. Furthermore, the expression of two viral genes, i.e. IE1 (Fig. 5B) and VP28 (Fig. 5C), were markedly decreased at 6 h after WSSV infection in Hpt cells if compared to that of the control group, which implied that the delivery of extra rCqCaspase protein showed negative effect on WSSV replication, possibly by the enhanced

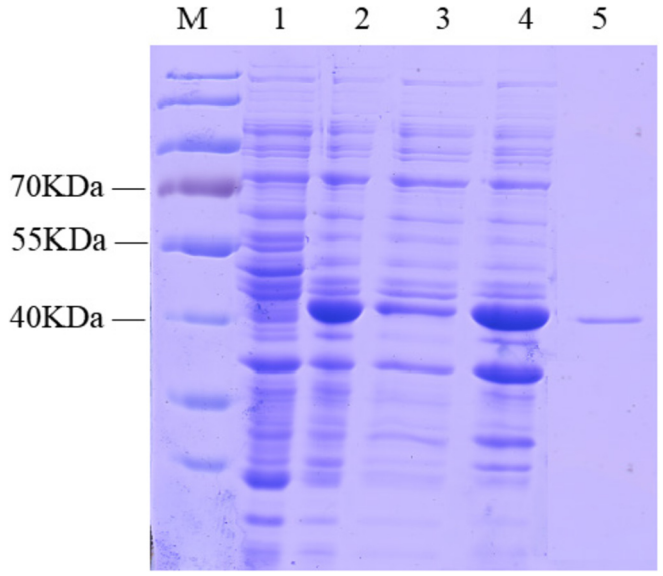

Fig. 4. The expression and purification of recombinant *CqCaspase* protein. SDS-PAGE analysis of rCqCaspase expression. The expression of rCqCaspase was induced by IPTG in *E.coli* (BL21:DE3). Lane M: protein molecular standard; 1: pET28a-CqCaspase recombinant clone, non-induced; 2: pET28a-CqCaspase recombinant clone, IPTG induced; 3: supernatant after sonication of rCqCaspase expressed as soluble protein; 4: precipitation after sonication of rCqCaspase expressed as inclusion body. 5: purified recombinant CqCaspase protein. rCqCaspase was purified by affinity chromatography with Ni Resin FF beads.

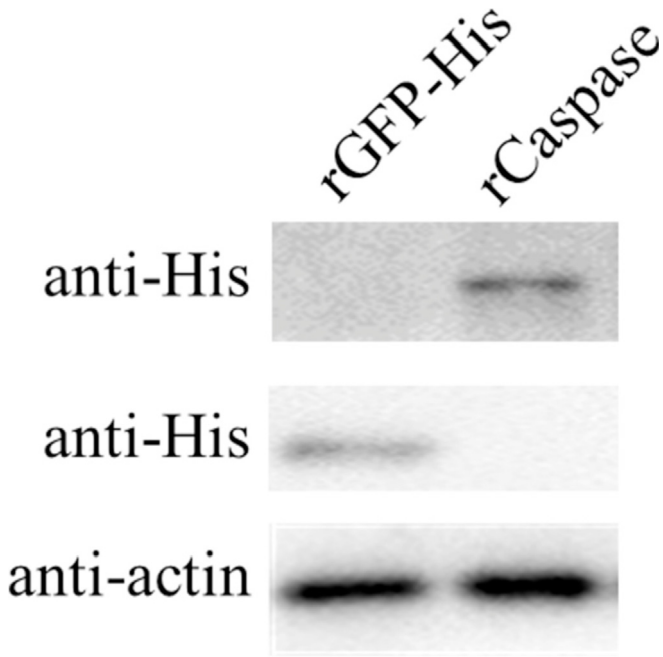

Fig. 5. Decreased WSSV replication by delivery of rCqCaspase protein into Hpt cells from red claw crayfish. (A): The anti-His antibody was used to detect rCqCaspase and rGFP-His after delivery of the recombinant proteins, respectively, into Hpt cells. The β-actin was taken as an internal control. (B-C): The transcription of IE1 and VP28 was reduced after delivery of rCqCaspase protein into Hpt cells. Hpt cells were delivered with extra-rCqCaspase followed by WSSV challenge for 6 h. The mRNA expression of IE1 and VP28 were examined by qRT-PCR and shown in B and C, respectively. The gene expression of both IE1 and VP28 exhibited significant decrease comparing to that of control groups post WSSV infection. The delivery of rGFP protein into cells was used as the control groups. The asterisk indicated the significant difference when compared with controls ($^{*}p < 0.05$, $^{**}p < 0.01$).

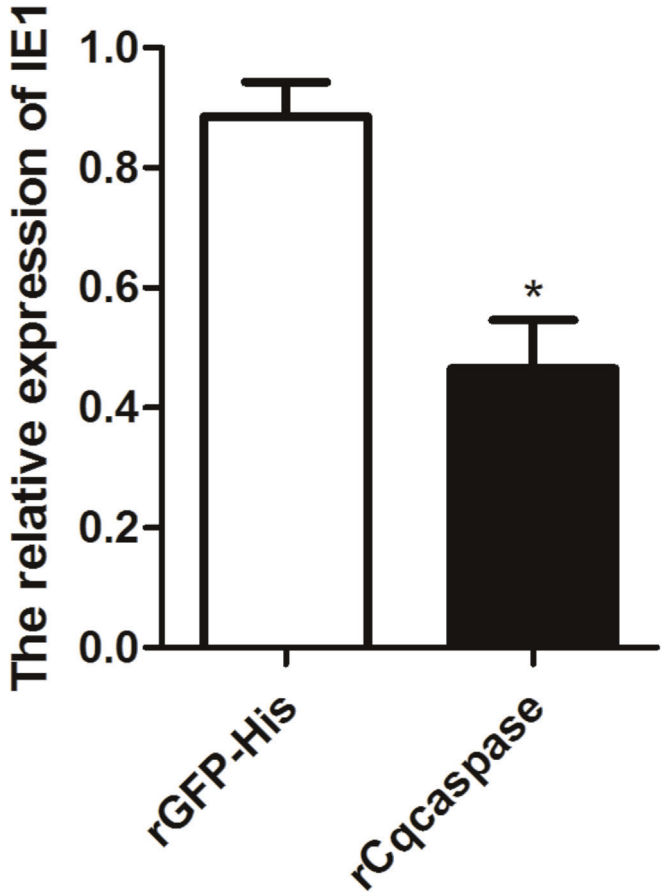

Fig. 5. (*continued*)

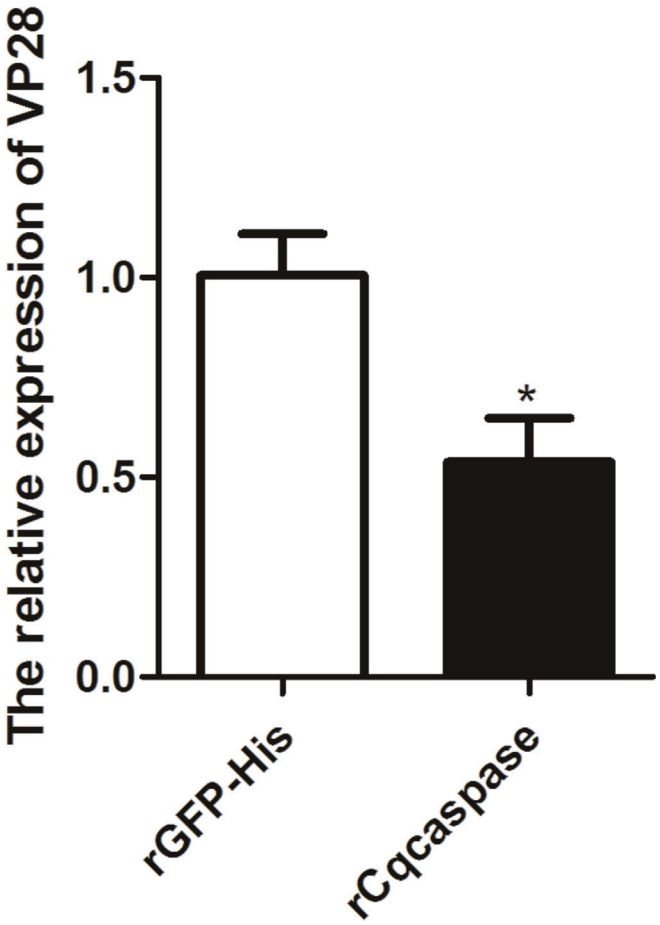

Fig. 5. (*continued*)

apoptotic activity, in Hpt cell cultures from *C. quadricarinatus*. Caspase has been reported to participate in antiviral response in shrimp. For instance, a *PjCaspase* protein was found to be required for shrimp antiviral apoptosis in *M. japonicus*. In addition, it was also reported that the gene silencing of *PjCaspase* resulted in the increase of virus copies (Wang et al., 2008). Moreover, when *PjCaspase* was activated by some small chemical molecules, like IL-2 and evodiamine, the mortalities of WSSV-infected shrimp was decreased in *M. japonicas*, suggesting that *PjCaspase* might play a role in anti-WSSV response via the enhancement of apoptotic activity (Zhi et al., 2011a). In our present study, the WSSV gene expression of both IE1 and VP28 were significantly decreased after the delivery of rCqCaspase protein in red claw crayfish Hpt cells, which was in consistent with the reduced WSSV replication by overexpression of the *PjCaspase* in *M. japonicus* (Zhi et al., 2011b). It has been reported that the virus-induced apoptosis was enhanced when the *PjCaspase* mRNA contained fragment 3 mRNA (mRNA3) was overexpressed in hemocytes. Furthermore, the WSSV copies were reduced after *PjCaspase* mRNA3 overexpression, which indicated that *PjCaspase* might play a role in anti-WSSV response via the enhancement of apoptotic activity. Therefore, we speculated that the recombinant protein rCqCaspase might mediate the apoptotic activity to defense against WSSV infection. However, no caspase activity was found in rCqCaspase, which might be due to the less catalytic activity caused by prokaryotic expression of recombinant *CqCaspase* (data not shown). Similar result has been found in *Drosophila*, in which the caspase-1 protein (named as DCP-1) expressed by *E.coli* was lacking of protease activity (Song et al., 1997). It is well known that apoptosis could remove unwanted and potentially dangerous cells such as virus-infected cells apoptosis (Hardwick, 2001; Hengartner, 2000). And it was reported that apoptosis was important in defense against WSSV infection (Wang et al., 2013b). Once infected by WSSV, the shrimp apoptosis-related genes like *PmCasp* was up-regulated and actively promoted apoptosis (Wang et al., 2008). Hence, we speculated that the recombinant protein rCqCaspase might promote caspase activity followed by the increased apoptotic activity, resulting in the inhibition of viral replication, within Hpt cells. Certainly, the mechanism of the inhibition on WSSV replication by *CqCaspase* in molecular details still needs furthermore investigations.

4. Conclusion

In conclusion, a caspase gene of *CqCaspase* was characterized from red claw crayfish *C. quadricarinatus*. Moreover, functional study indicated that r*CqCaspase* could significantly inhibit the WSSV replication in crayfish Hpt cells. Therefore, these data shed new light of the mechanism of WSSV infection and provide a strategy for WSSV disease control in crustacean aquaculture.

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