Fish & Shellfish Immunology 57 (2016) 213–221

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Identification of an anti-lipopolysacchride factor possessing both antiviral and antibacterial activity from the red claw crayfish *Cherax quadricarinatus*

Feng-yu Lin [a], Yan Gao [a], Hao Wang [a], Qiu-xia Zhang [a], Chang-lin Zeng [a], Hai-peng Liu [a, b, *]

[a] State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, Fujian, PR China
[b] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources (Xiamen University), State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen 361102, Fujian, PR China

ARTICLE INFO

Article history:
Received 6 July 2016
Received in revised form
10 August 2016
Accepted 14 August 2016
Available online 17 August 2016

Keywords:
Anti-lipopolysacchride factor
Cherax quadricarinatus
Antiviral activity
Antimicrobial peptide

ABSTRACT

It is well-known that anti-lipopolysacchride factors (ALFs) are involved in the recognition and elimination of invading pathogens. In this study, the full-length ALF cDNA sequence of the red claw crayfish *Cherax quadricarinatus* (termed *CqALF*) was cloned from a suppression subtractive hybridization library constructed using red claw crayfish hematopoietic tissue cell (Hpt cell) cultures following challenge with white spot syndrome virus (WSSV). The full-length cDNA sequence of *CqALF* was 863 bp, and the open reading frame encoded 123 amino acids with a signal peptide in the N-terminus and a conserved LPS-binding domain. Unlike most ALFs, which are highly expressed in haemocytes, high expression levels of *CqALF* were detected in epithelium, the stomach and eyestalks, while lower expression was detected in Hpt, nerves, the heart, muscle tissue, gonads, haemocytes, intestines, gills and the hepatopancreas. To further explore the biological activities of *CqALF*, mature recombinant *CqALF* protein (rCqALF) was expressed and purified using a eukaryotic expression system, and an antimicrobial activity test was carried out. rCqALF clearly exerted antiviral activity, as evidenced by the severe disruption of the envelope of intact WSSV virions following co-incubation of virions with rCqALF. Additionally, pre-incubation of WSSV with rCqALF resulted in both a significant reduction in WSSV replication in red claw crayfish Hpt cell cultures and an increased survival rate among animals. Furthermore, rCqALF was effective against both Gram-negative bacteria and Gram-positive bacteria, particularly *Shigella flexneri* and *Staphylococcus aureus*. A membrane integrity assay suggested that rCqALF was unlikely to disrupt bacterial membrane integrity compared to cecropin P1. Taken together, these data suggest that *CqALF* may play an important role in immune defence in the crustacean *C. quadricarinatus*.

1. Introduction

Antimicrobial peptides (AMPs) are small cationic molecules that are ubiquitously found in all kingdoms of living organisms and are among the first lines of immune defence against microbial invasion [1]. Unlike traditional antibiotics, AMPs rarely result in bacterial drug-resistance [2]. Therefore, as potentially bioactive substances, AMPs are expected to replace traditional antibiotics. To date, many AMPs, including astacidin [3], crustin [3–5] and anti-lipopolysacchride factor (ALF) [6], have been characterized from crayfish species. ALF is a cationic AMP capable of binding and neutralizing lipopolysaccharide (LPS) [7] and is a crucial bioactive substance in crustacean innate immunity. Since ALF was first identified from the American horseshoe crab *Limulus polyphemus* [8], numerous ALFs have been found in other crustaceans such as *Pacifastacus leniusculus*, *Litopenaeus vannamei*, *Fenneropenaeus chinensis*, *Homarus americanus* and *Scylla paramamosain* [6,9–12]. Although different ALF isoforms possess low similarity, they exhibit a common feature: the LPS-binding domain, a disulphide loop formed by two conserved cysteine residues, which is considered to be the key functional domain for ALF antibacterial activity [7]. Both recombinant ALF proteins and synthetic peptides of the LPS-binding domain exhibit different antimicrobial activities against Gram-positive and Gram-negative bacteria [13,14]. In addition to

* Corresponding author. State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361102, Fujian, PR China.
E-mail address: Haipengliu@xmu.edu.cn (H.-p. Liu).

http://dx.doi.org/10.1016/j.fsi.2016.08.037
1050-4648/

antibacterial activity, ALF antiviral activities have also been reported. For instance, gene silencing of ALF in *P. leniusculus* via RNA interference specifically resulted in higher rates of white spot syndrome virus (WSSV) replication [6]. A synthesized LPS-binding domain of *L. polyphemus* was able to inhibit the entry of HIV-1, HCV and HSV1 into host cells [15]. The recombinant protein ALFPm3 exerts antiviral activity by interacting with WSSV structural proteins [16]. All of these data suggest that ALFs play important roles in immune defence against microbial infection. Thus, further functional characterization of *CqALF* in terms of antimicrobial activity, particularly antiviral properties, will bring new insight for disease control in aquaculture.

The red claw crayfish *C. quadricarinatus*, introduced to China from Australia in the 1990s, is an important aquaculture crustacean species with high economic value. It has been reported that red claw crayfish can be infected by WSSV, a mortal pathogen of both shrimp and crayfish aquaculture, and crayfish Hpt cell cultures are a good cell model for the study of WSSV infection [17,18]. Previously, a partial sequence of *CqALF* was screened from a suppression subtractive hybridization (SSH) library constructed from hematopoietic tissue (Hpt) cell cultures of *C. quadricarinatus* post- WSSV challenge [17]. Investigation of the role of *CqALF* in immunity, particularly its anti-WSSV activity, will be useful for WSSV disease control in aquaculture. In the present study, the full-length cDNA sequence of *CqALF* and its gene expression profiles in different tissues were investigated. Furthermore, recombinant *CqALF* protein (rCqALF) was expressed and purified with a eukaryotic expression system and assessed against both WSSV and bacteria in an antimicrobial activity test. The results indicated that *CqALF* may play an important role in the innate immune defence against microbial infection in the crustacean *C. quadricarinatus*.

2. Materials and methods

2.1. Animals and tissue collection

Healthy *C. quadricarinatus* crayfish, averaging 48 ± 5 g in body weight, were obtained from the Tenglong Company, Xianyou, Fujian Province, China, and acclimatized in aerated freshwater at 26 °C for one week before sample collection. Haemocytes were obtained with a sterile syringe and centrifuged for 10 min at 1000*g* (4 °C). Other tissues (stomach, gonad, muscle, nerve, intestine, heart, Hpt, hepatopancreas, gill, epithelium and eyestalk) were sampled from three random individuals for total RNA isolation.

2.2. RNA extraction and cDNA synthesis

Total RNA from tissues (described above) was isolated using Trizol reagent (Roche, Mannheim, Germany) according to the manufacturer's instructions. RNase-Free DNase I (Ambion, Austin, Texas, USA) was used to eliminate genomic DNA contamination in the extracted RNA following the manufacturer's protocol. The extracted RNA was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and analysed by 1.0% agarose gel electrophoresis. Then, cDNA was synthesized using a PrimeScript™ RT Reagent Kit (TaKaRa) according to the manufacturer's instructions.

2.3. Gene cloning of the full-length CqALF cDNA

A partial *CqALF* cDNA sequence of *C. quadricarinatus* was isolated from an SSH library upon WSSV infection in our previous study [17]. To obtain the full-length cDNA sequence of *CqALF*, 5′- and 3′-RACE were carried out using a SMART RACE cDNA Amplification Kit (Clontech, Madison, Wisconsin, USA) following the manufacturer's protocol. The gene-specific primers for RACE, RACE3F and RACE5R, are shown in Table 1. The PCR conditions were as follows: 5 min at 94 °C; 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s; and 72 °C for 7 min. All amplified PCR products were gel-purified using a Gel Extraction Kit (Dongsheng Biotech, Co., Ltd., Beijing, China), and the expected DNA fragments were ligated into a PMD18-T vector (TaKaRa). The vectors were transformed into competent *E. coli* DH5α cells, and the recombinants were identified via resistance selection on ampicillin-containing LB plates incubated overnight at 37 °C. Bacterial colony PCR was performed to screen positive colonies as previously described [19]. The positive clones were picked for sequencing at Shanghai Genewindows Biotech Co. Ltd (China). Then, the full-length cDNA sequence of *CqALF* was assembled using SeqMan software.

2.4. Bioinformatics analysis

The *CqALF* open reading frame (ORF) and amino acid sequence were analysed using the ExPASy translate tool (http://web.expasy.org/translate/). Protein sequences homologous to *CqALF* were identified by BLAST searching (http://blast.ncbi.nlm.nih.gov/Blast). The signal peptide was predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The conserved domain was identified using the Conserved Domain database of NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/). Putative glycosylation site analysis was carried out using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). An ALF phylogenetic tree was constructed with Mega 6.06 using the Neighbour-Joining method based on sequence alignments of *CqALF* with other homologous amino acid sequences.

2.5. CqALF transcript distribution in various tissues

As mentioned earlier, twelve tissues were dissected from three random healthy crayfish and prepared for total RNA isolation and cDNA synthesis as described above. To determine the *CqALF* mRNA transcript levels in the different tissues, qRT-PCR was performed using an ABI PCR machine (Applied Biosystems 7500, UK). A pair of specific primers (Q-F/Q-R in Table 1) was designed using Primer Premier 5.0 to amplify a target product from the cDNA, and the crayfish 16S ribosomal gene (GenBank: AF135975.1) was employed as an internal standard. *CqALF* expression levels relative to 16S gene expression were quantified using the $2^{-\Delta\Delta Ct}$ method [20]. The primers designed for 16S gene amplification (16S-F and 16S-R) are shown in Table 1. qRT-PCR reactions were performed in a 96-well PCR plate. The reaction volume consisted of 20 μl of mixture containing 10 μl of SYBR Green Master (2×) (Roche, USA), 0.5 μl of each primer (10 μM), 5.0 μl of cDNA, and 4 μl of sterile water. The qRT-PCR program was as follows: 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. The amplification specificities of *CqALF* and 16S were confirmed by

Table 1
Primers used in the experiments.

Primers	Sequences (5′–3′)
RACE3F	ATCTGTCGCTTGTTGCTGAGTG
RACE5R	GAGAAGCATCAACGCGGAGC
16S-F	AATGGTTGGACGAGAAGGAA
16S-R	CCAACTAAACACCCTGCTGATA
Q-F	GCTGGACTGTGGGATAATGGC
Q-R	GATGCTTCTCCTCTGATGGTGGT
E-F	CCGGAATTCCAGATTACAGAGGCTCTGG
E-R	GCTCTAGATTAATGATGATGATGATGGTGAGTTTTCAAAAAATCTGTTGC
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC

analysing the melt curves following PCR reactions. Reactions were performed in triplicate for each sample.

2.6. Recombinant expression and purification of rCqALF in the yeast Pichia pastoris

To further characterize the biological activities of the *CqALF* protein, we expressed the recombinant *CqALF* protein in a *P. pastoris* yeast expression system [21]. The specific primers E-F and E-R, shown in Table 1, to amplify the ORF of *CqALF* were designed to contain *EcoR* I and *Xba* I restriction sites (underlined), respectively. In the reverse primer E-R, the bases TTA and 6-histidine were also introduced. PCR was carried out, and PCR products were gel-purified. Then, the expected PCR product and the expression vector pPICZαA (Invitrogen) were digested with the enzymes *EcoR* I and *Xba* I (TaKaRa). The digested PCR product was subsequently ligated into the pPICZαA vector. Next, the recombinant plasmid pPICZαA-*CqALF* was linearized by the enzyme *BamH* I before transformation into the host *P. pastoris* GS115 via electroporation. After incubation on yeast extract peptone dextrose medium for 68 h at 28 °C, positive clones were screened, and recombinant protein expression was induced by 1% methanol every 24 h. To assess protein expression, the protein-containing supernatant was analysed by 15% SDS-PAGE. A Ni-NTA agarose column was used to purify *CqALF* with a 6-histidine tag as previously described [22]. Briefly, supernatant was separated from the culture media and dialysed against 50 mM PBS buffer (pH 8) for 36 h, then loaded onto a Ni-NTA agarose column after filtration through a 0.45-μM filter membrane. The bound recombinant protein was eluted using elution buffer containing 300 mM imidazole on an AKTApurifier UPC 100 (GE, USA). Purification quality was analysed by 15% SDS-PAGE, and the purified *CqALF* recombinant protein was measured using a pierce™ BCA protein assay kit (Thermo Scientific, USA) with BSA as the protein standard. To further confirm *CqALF* expression, corresponding SDS-PAGE protein bands were assessed using MALDI-TOF/TOF mass spectrometry.

2.7. In vitro antiviral activity assays

WSSV was kindly provided by Prof. Xun Xu (The Third Institute of Oceanography, Xiamen, China) and was confirmed using absolute quantitative PCR. Integral WSSV viral particles were isolated from infected crayfish tissues as previously reported [23]. The effects of rCqALF on the WSSV envelope were investigated by performing transmission electron microscopy (TEM) analysis using a cryogenic transmission electron microscope (FEI Tecnai G2 Spirit BioTwin). This assay was performed by incubating 10^8 copies of virions with two concentrations of rCqALF (20 and 40 μM) for 30 min at room temperature, followed by TEM examination. Bovine serum albumin (BSA) was used as the negative control protein. Briefly, WSSV virions were fixed with 1% paraformaldehyde for 20 min, mounted onto carbon-stabilized copper grids, negatively stained with 2% sodium phosphotungstate for 5 min followed by natural drying at room temperature for at least 3 h, and subsequently subjected to TEM analysis.

qRT-PCR was performed to determine whether rCqALF exerted an effect on the replication of WSSV in Hpt cell cultures. Hpt cell cultures from *C. quadricarinatus* were prepared in a 24-well plate as previously described in a study by Liu et al. [17]. The assay was conducted by pre-incubating 5×10^5 copies of WSSV virions/well with rCqALF (20 μM) for 30 min at 25 °C. Then, the mixture was added into Hpt cell culture (5×10^5 cells/well). Total RNA was extracted from the Hpt cell cultures at 6 h post-WSSV infection for qRT-PCR. qRT-PCR was carried out to analyse the expression of the immediate early gene (IE1) of WSSV using the 16S gene as an internal standard. The specific primer pair for IE1 (IE1-F and IE1-R) amplification is shown in Table 1. The assay was performed at least three times.

2.8. rCqALF-induced protection against WSSV infection in red claw crayfish

To determine whether rCqALF exerted a protective role against WSSV, red claw crayfish (12 ± 2 g) were infected by injecting WSSV pre-incubated with rCqALF, and the survival rate was calculated. The animals were randomly divided into a rCqALF group and a BSA control group. In the rCqALF group, rCqALF (20 μM) was co-incubated with WSSV for 30 min at 25 °C; then, the mixture was resuspended in sterile crayfish saline buffer (CFS: 0.2 M NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 2 mM $NaHCO_3$, pH 6.8) at a concentration of 1 WSSV virion copy per microliter. Each crayfish was injected with 100 μl of WSSV suspension in the fourth walking leg. BSA (20 μM) was used as the negative control protein for the control group. Thirty-two crayfish were employed per treatment. The percent survival of both groups was recorded every 12 h.

2.9. Antibacterial activity assays

The antibacterial activity of rCqALF was evaluated against seven Gram-positive bacteria (*Micrococcus lysodeikticus Fleming, Streptococcus agalactiae*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Corynebacterium glutamicum, Bacillus subtilis,* and *Staphylococcus aureus*), twelve Gram-negative bacteria (*Pseudomonas stutzeri*, *Edwardsiella tarda*, *Vibrio parahemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi*, *Vibrio fluvialis*, *Aeromonas hydrophila*, *Escherichia coli*, *Pseudomonas fluorescens*, *Shigella flexneri, Escherichia coli* MC1061) and one yeast (*Candida albicans*). The Minimal Inhibition Concentration (MIC) was determined by performing a liquid growth inhibition assay as previously described [13] and calculated as the lowest rCqALF concentration yielding no detectable growth of bacteria and yeast. The Minimal Bactericidal Concentration (MBC) was calculated as the lowest concentration of rCqALF that killed the microorganisms after incubation for 48 h. Briefly, rCqALF was dissolved in MilliQ water to obtain 2-fold serial dilutions from 1.375 μM to 44 μM for the MIC and MBC assays. This assay was performed in triplicate.

2.10. Membrane integrity assay

To examine the mechanism underlying the effects of rCqALF on bacteria, a whole-cell real-time membrane integrity assay was performed employing *E. coli* MC1061, which constitutively expresses luciferase, as described in previous studies [13,24,25]. Perforation of the *E. coli* cell membrane led to an influx of externally applied D-luciferin into the luciferase-expressing cells and caused light emission. This assay was performed in a white 96-well plate. Briefly, 50 μL of Mueller-Hinton Broth medium containing 1×10^7 CFU *E. coli* MC1061 cells and 2 mM D-luciferin potassium salt (Sigma, USA) was mixed in each well with 50 μL of diluted *CqALF* protein with a final concentration of 2.75 μM, 5.5 μM or 44 μM. The cecropin P1 protein (1 μM) (Sigma, USA) and argireline (25 μM) (Tash, China) were employed as a positive control peptide and a negative control peptide, respectively. Luminescence was monitored using an Infinite M200 (Tecan, Switzerland). All measurements were performed in triplicate.

2.11. Statistical analysis

The data were analysed by Student's *t*-test and presented as the mean ± SD from three independent assays for comparison between

two groups using SPSS ver. 18.0 software at significance levels of 5% and 1%. Differences in survival percentage between the groups were analysed by Kaplan–Meier log-rank X^2 tests.

3. Results and discussion

3.1. Characterization of the full-length cDNA sequence of CqALF

The full-length cDNA sequence of *CqALF* was determined using the RACE method (GenBank accession no. KX083340). As shown in Fig. 1, the cDNA of *CqALF* was 863 bp in length, including a 5′-untranslated region (UTR) of 108 bp, an open reading frame of 372 bp that encoded 123 amino acids, and a 3′-UTR of 383 bp. SignalP analysis predicted that *CqALF* possessed a 25-amino-acid signal peptide at the N-terminus. The calculated protein molecular weight was 13.35 kDa with an estimated theoretical isoelectric point of 9.51, which was highly similar to ALF from *P. leniusculus* [6]. Sequence alignments of *CqALF* with other homologs showed that the *CqALF* gene contains two conserved cysteine residues and a clustering of positive charges within the disulfide loop (Fig. 2), which has been defined as the putative LPS-binding domain [7]. The deduced amino acid sequences of *CqALF* demonstrated similarities of 61, 58, 56, 44, 39 and 36% to ALF of *P. clarkii* (GenBank ID ADX60063.1), *Marsupenaeus japonicus* (GenBank ID AME17862.1), *Penaeus monodon* (GenBank ID AER45468.1), *P. leniusculus* (GenBank ID ABQ12866.1), *Eriocheir sinensis* (GenBank ID ADZ46233.1) and *L. polyphemus* (GenBank ID 1307201A), respectively. Phylogenetic analysis of *CqALF* with other homologs suggested that *CqALF* was similar to other crustacean ALFs (Fig. 3) and revealed that *CqALF* was clustered much closer to shrimp, lobster and red swamp crayfish than that of horseshoe crab.

3.2. The transcript distribution of CqALF in various tissues

CqALF mRNA was expressed in all tissues examined from the healthy red claw crayfish. High mRNA expression levels of *CqALF* were observed in the stomach, epithelium and eyestalks, while low expression levels in Hpt, the heart, nerves, gonads, muscle tissue, haemocytes, intestines, gills and the hepatopancreas (Fig. 4). Generally, AMPs are primarily distributed in the haemocytes or released into the plasma from haemocytes upon microbial challenge in crustaceans [26]. However, *CqALF* mRNA transcripts could be detected in all twelve of the examined tissues, although with very low expression in haemocytes, which was not in agreement with the ALF mRNA expression profiles of other crustaceans such as *ALFSp*, *Sp-ALF1* and *Sp-ALF2* in *Scylla paramamosain* [12,13], *PtesALF1* and *PtesALF1* in *Portunus trituberculatus* [27] and *SsALF* in *Scylla serrata* [28]. This differential expression indicates that *CqALF* may possess other unknown functions in the defence against microbial invasion in the red claw crayfish. The epithelial tissue is one of the first-line physical barriers protecting crustaceans against microbial invasion. The high expression levels of *CqALF* in epithelial tissue imply that *CqALF* may play an important role in immune protection against microbes invading through this physical barrier. The high mRNA transcripts observed in the stomach, a pivotal digestive tissue, indicate that *CqALF* likely significantly contributes to antimicrobial activity against pathogens invading via oral infection. The eyestalk is a crucial neuroendocrine organ and the

```
          10         20         30         40         50         60
1   GTACGCGGGGATCACACCACACCACACAAGTGCTTCACAGAGCTTACACTCTACCAAGGT
          70         80         90        100        110        120
61  GCTCCAAGACTCACACTTGAGTCTTGAAGACAAAACTTAGAAGTAAAGATGCGGTCGTCT
                                                    M  R  S  S
         130        140        150        160        170        180
121 GTGTTAGTGAGCGTGGTGGCAGTGTCGCTGCTGGTGGCACCTCTAGTGCCACAGAGCAAC
41   V  L  V  S  V  V  A  V  S  L  L  V  A  P  L  V  P  Q  S  N
         190        200        210        220        230        240
181 GCACAGATTACAGAGGCTCTGGTAACAGCCTTCGCTGGGAAAGTTGCTGGACTGTGGGAT
61   A  Q  I  T  E  A  L  V  T  A  F  A  G  K  V  A  G  L  W  D
         250        260        270        280        290        300
241 AATGGCCAGCTGGAGCTACTGGGTAACTACTGTAACTACAACGTCAGGCCTACCATCAAG
81   N  G  Q  L  E  L  L  G  N  Y  C  N  Y  N  V  R  P  T  I  K
         310        320        330        340        350        360
301 AAGTTTCAGCTATACTTCAATGGAAGCATGTATTGCCCTGGCTGGACCACCATCAGAGGA
101  K  F  Q  L  Y  F  N  G  S  M  Y  C  P  G  W  T  T  I  R  G
         370        380        390        400        410        420
361 GAAGCATCAACGCGGAGCAGGTCTGGTGTGGTCGGGGAGACCACCAAGGACTTCATCAGG
121  E  A  S  T  R  S  R  S  G  V  V  G  E  T  T  K  D  F  I  R
         430        440        450        460        470        480
421 AAGGCTATGGCAGCCGGTCTCATCACTCAGCAACAAGCAACAGATTTTTTGAAAACTTAA
141  K  A  M  A  A  G  L  I  T  Q  Q  Q  A  T  D  F  L  K  T  *
         490        500        510        520        530        540
481 GTCATTTCTCTTTCTTAGAACCCATAAATAACCCGTATTTTGGGGACAACGAGCAGCTGT
         550        560        570        580        590        600
541 ATTTCATAGTTCCCCCGAGTTATCTGGAAAAATAATACGTTTTCGTGGTTCAAAGAATAG
         610        620        630        640        650        660
601 TTTAATTTCTTATATCTGATTAAGATTCTTCAACTGATAATTAAATTTTTGTTCAGATTC
         670        680        690        700        710        720
661 CCAAATATTTGTCACAGACAACTAAGGAACCACGTGATCGTGATTCTCTTACCATTCTGC
         730        740        750        760        770        780
721 TTATTTACTTCTGTGATCACATGCTCAAGATATTTTTTATATTTGGTAGTCATTCCAAAT
         790        800        810        820        830        840
781 GAATGCCTTATATAATGTTTTCGTGATATGTAATAAACAAATTTAATACATTCGTAAAAA
         850        860
841 AAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. The full-length cDNA sequence and the deduced amino acid sequence of *CqALF* from the red claw crayfish *C. quadricarinatus*. The start codon (ATG) and stop codon (TAA) are in red. The putative signal peptide sequence is shaded. The putative LPS-binding domain is underlined. The asterisk indicates the deduced stop codon. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

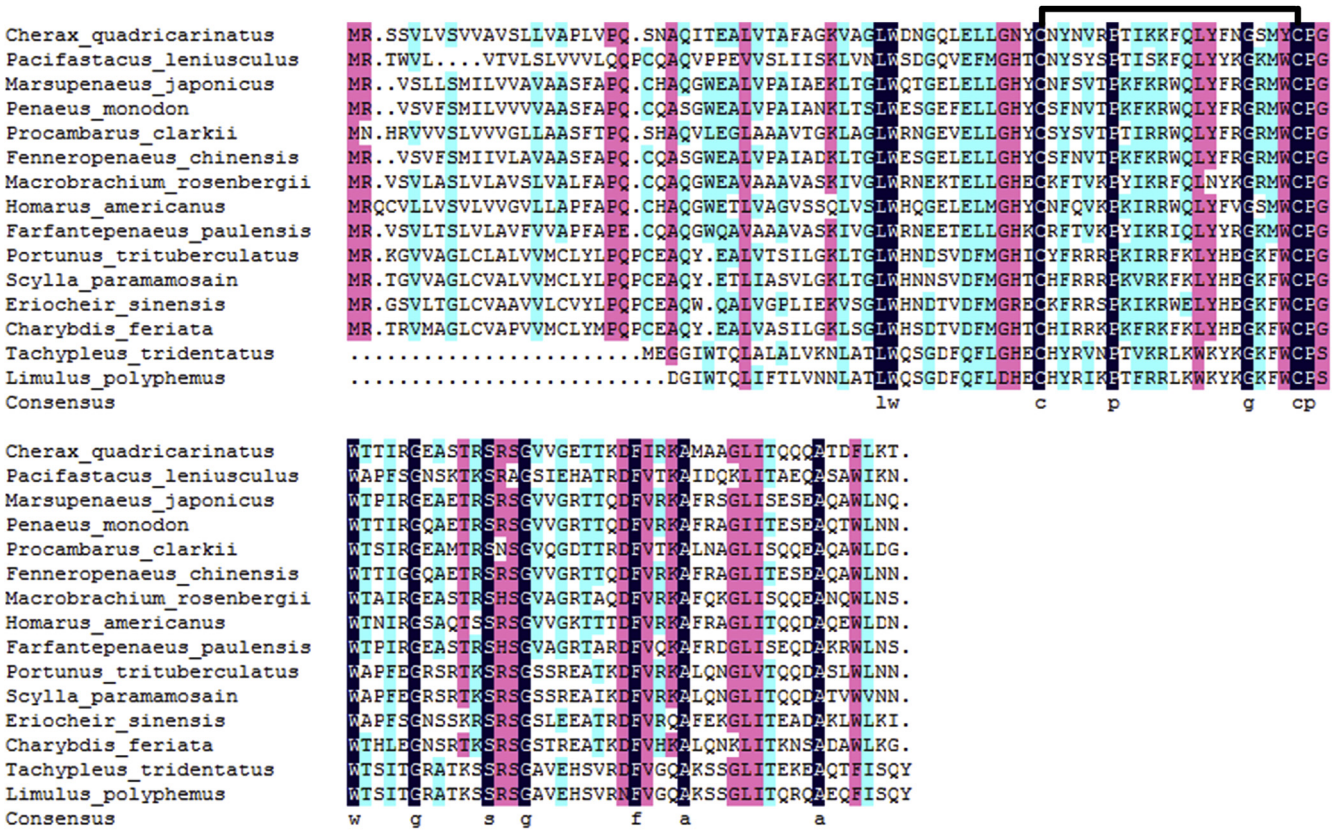

Fig. 2. Amino acid sequence alignment of *CqALF* with other known ALFs: *Penaeus monodon* (AER45468.1); *Fenneropenaeus chinensis* (AFU61125.1); *Marsupenaeus japonicus* (AME17862.1); *Homarus americanus* (ACC94269.1); *Procambarus clarkii* (ADX60063.1); *Macrobrachium rosenbergii* (AEP84102.1); *Farfantepenaeus paulensis* (ABQ96193.1); *Pacifastacus leniusculus* (ABQ12866.1); *Eriocheir sinensis* (ADZ46233.1); *Charybdis feriata* (AKF40834.1); *Portunus trituberculatus* (C0KJQ4.2); *Scylla paramamosain* (AFI43796.1); *Tachypleus tridentatus* (AAK00651.1); and *Limulus polyphemus* (1307201A). The two conserved cysteines forming putative a disulphide bond are linked by a black line.

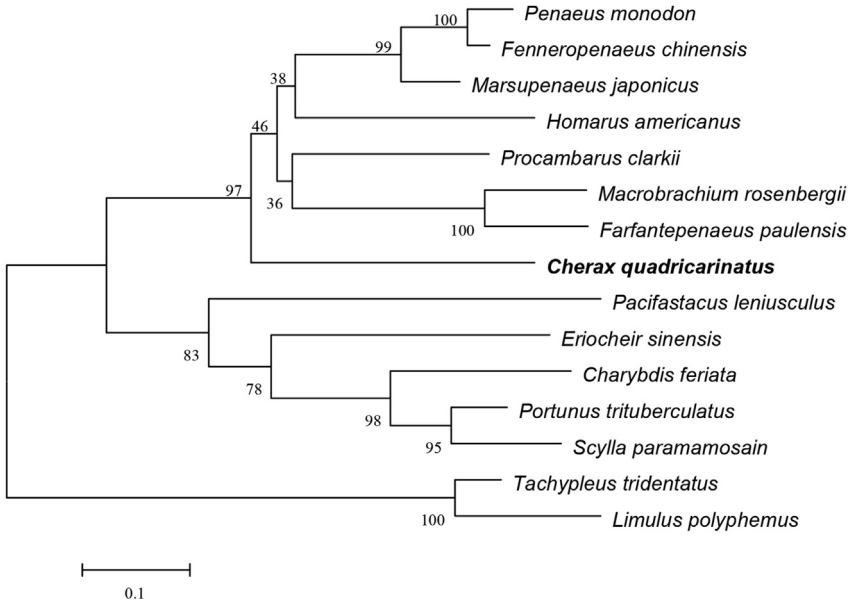

Fig. 3. Phylogenetic analysis of *CqALF* (bold) and other known ALFs. The values on each branch represent the bootstrap values (%) for 1000 replications, and the bars indicate the distance.

major source of hormones in crustaceans [29]. It was previously reported that three ALF isoforms identified from an eyestalk cDNA library in *P. trituberculatus* might confer protective effects in the eyestalk [27]. The high expression levels of *CqALF* imply that the eyestalk may be involved in the regulation of immune defence. Taken together, these data imply that *CqALF* is likely a key factor for

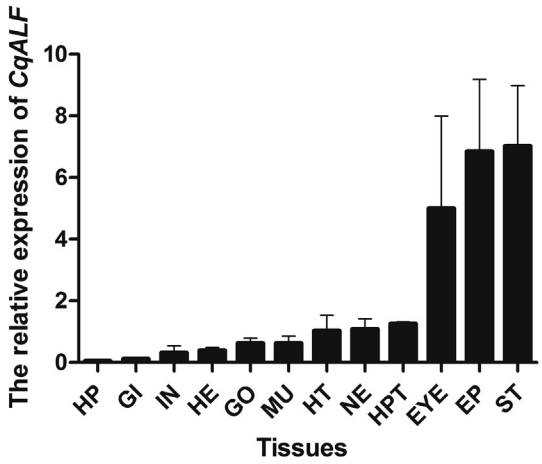

Fig. 4. The mRNA expression profiles of *CqALF* in different tissues. HP: hepatopancreas, IN: intestine, HE: haemocytes, NE: nerve, GI: gill, GO: gonad, MU: muscle, EP: epithelial tissue, Hpt: hematopoietic tissue, ST: stomach; EYE: eyestalk. The relative transcript levels of *CqALF* in muscle were employed as the calibrator (value set as 1). The experiment was repeated three times. Bars represent ± standard error of the mean.

immune protection in the red claw crayfish.

3.3. Recombinant expression and purification of rCqALF

To explore the biological activities of the *CqALF* protein, a mature *CqALF* peptide was recombinantly expressed with the pPICZaA vector in the yeast *P. pastoris* and purified via single-step purification using a Ni-NTA affinity column. The purified rCqALF was approximately 11 kDa in size, which was in agreement with the calculated molecular mass of the mature peptide. The rCqALF protein showed an additional band of approximately 14 kDa (Fig. 5). We speculate that putative glycosylation might occur in *CqALF* during recombinant protein expression in the eukaryotic expression system, as reported previously [13]. Prediction of putative glycosylation sites via bioinformatics analysis revealed a putative glycosylated asparagine in the deduced amino acid sequence of *CqALF* at position 71. The two rCqALF protein bands observed by SDS-PAGE were also confirmed by MALDI-TOF/TOF mass spectrometry analysis, which demonstrated that several peptide fragments corresponded to the deduced protein sequences of *CqALF* (data not shown).

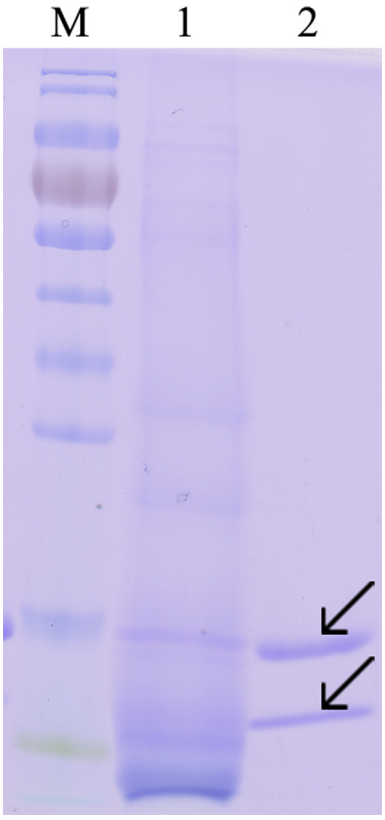

Fig. 5. SDS–PAGE analysis of rCqALF expression and purification. Lane M: molecular weight marker; lane 1: cultured medium from the pPICZαA/*CqALF* recombinant clone before protein purification; lane 2: purified rCqALF protein.

3.4. Antiviral activity of rCqALF

Previous studies have suggested that ALFs possess antiviral activity against WSSV [6,12,30,31]. However, the mechanism underlying the antiviral activity of ALFs still remains unknown. Intact WSSV virions are enveloped and elliptical in shape upon TEM examination following negative staining with paraformaldehyde [32], while non-enveloped WSSV virions with lower infectivity are thinner and longer than the intact virions and have a segmented appearance [33,34]. In the present study, the effect of rCqALF on WSSV structure was determined by performing TEM analysis of WSSV virions pre-incubated with rCqALF protein for 30 min. Compared to WSSV pre-incubated with 20 μM BSA protein or untreated by protein, the presence of 20 μM or 40 μM rCqALF protein significantly disrupted the viral envelope integrity of WSSV. As shown in Fig. 6, most of the WSSV virions that were pre-incubated with rCqALF were in a non-intact state, demonstrating no viral envelope when compared to WSSV virions in the control groups. It was previously reported that crayfish Hpt cell culture is a good cell model to study WSSV infection [35]. To further investigate whether disruption of WSSV envelope integrity caused by the interaction with rCqALF could affect WSSV replication, red claw crayfish Hpt cell culture was employed to explore the effects of rCqALF on WSSV replication in our present study. Notably, a significant reduction in IE1 expression was detected when cultured Hpt cells were infected with WSSV pretreated with rCqALF at 6 hpi when compared to the control group (Fig. 7), implying that rCqALF exerted a negative effect on viral replication, likely by interfering with the viral envelope proteins; these results are in agreement with the observation that WSSV replication was clearly inhibited by crab rSp-ALFs [13]. To further investigate whether rCqALF could protect the red claw crayfish against WSSV infection, animals were infected with WSSV pre-incubated with rCqALF, and the survival rate was calculated. As shown in Fig. 8, the survival rate of the rCqALF-treated group was significantly higher than that of the control group, demonstrating that rCqALF conferred enhanced protection against WSSV infection in the red claw crayfish. In a previous study, ALFPm3 in *P. monodon* exhibited antiviral activity by interacting with the structural proteins of WSSV as assessed in an *in vitro* pull-down assay [16], and FcALF5 in the Chinese shrimp *F. chinensis* exhibited antiviral activity by acting upon VP24, the major envelope protein of WSSV [36]. It was also reported that antiviral cationic peptides demonstrate antiviral activity by interacting with viral virions and directly or indirectly inhibiting viral replication [37]. Our present study suggests that rCqALF exerts antiviral activity by direct interacting with WSSV envelope proteins. These observations also reveal that the envelope proteins of WSSV play key roles in WSSV infection and replication. Taken together, these findings imply that rCqALF may bind to WSSV virions and disrupt their envelope integrity, which then leads to decreased viral infectivity.

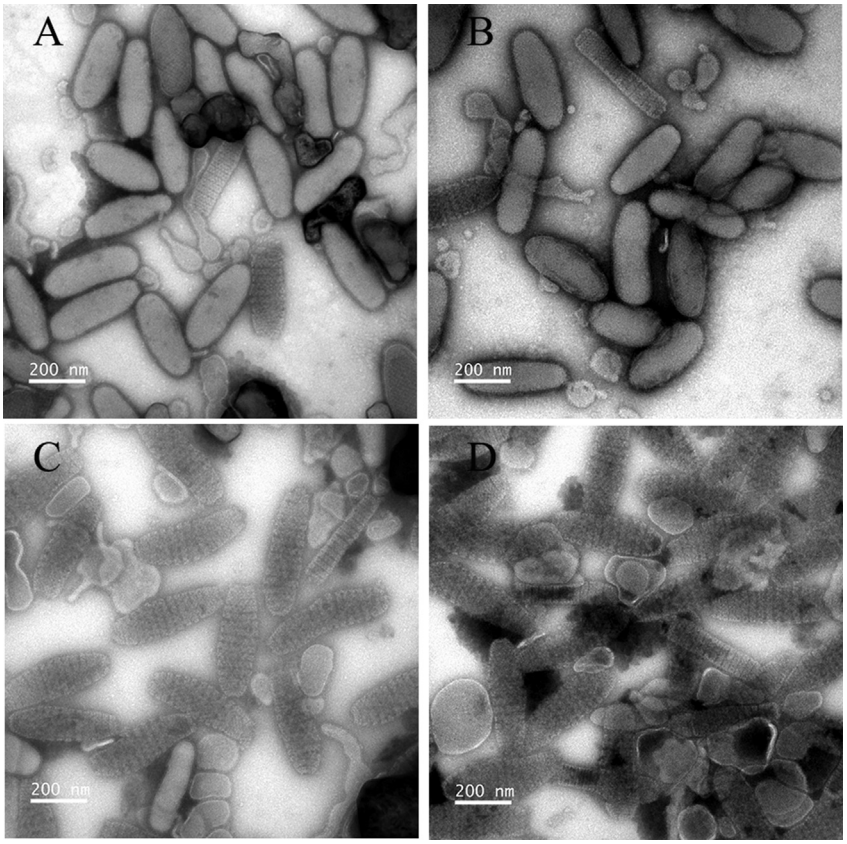

Fig. 6. Transmission electron micrograph of the WSSV envelope following incubation with different proteins. The WSSV virions were pre-incubated with MilliQ water (A), 20 μM BSA (B), 20 μM rCqALF (C) and 40 μM rCqALF (D), respectively. Bar, 200 nm.

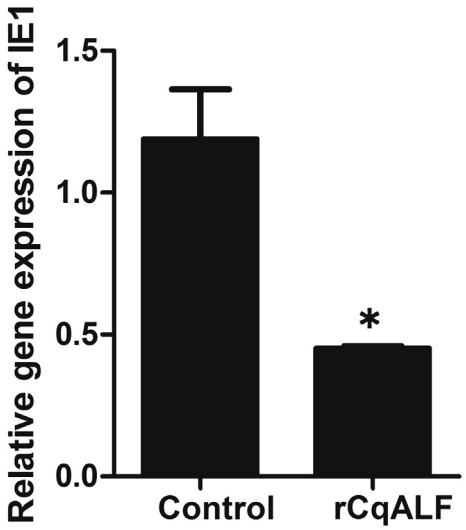

Fig. 7. Inhibited replication of WSSV by rCqALF in Hpt cell cultures. Transcript levels of the immediate early gene of WSSV (IE1) were reduced following pretreatment of WSSV with rCqALF as determined by quantitative RT-PCR. Hpt cell cultures were infected with WSSV pretreated with different proteins for 6 h. Control column: Hpt cells incubated with a WSSV mixture containing BSA (20 μM). rCqALF column: Hpt cells incubated with a WSSV mixture containing rCqALF (20 μM). This assay was repeated three times. Bars represent ±standard error of the mean. The asterisk indicates significant difference compared with control ($P < 0.05$).

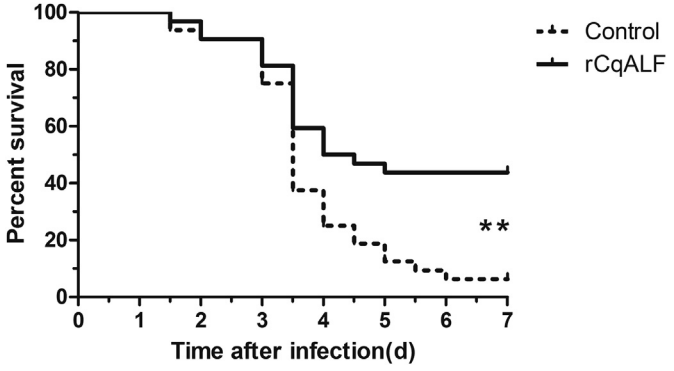

Fig. 8. Protection of red claw crayfish against WSSV infection by rCqALF. Infection of WSSV pretreated with rCqALF resulted in a significantly higher survival rate following viral infection when compared to infection by WSSV pretreated with BSA ($P < 0.01$).

3.5. Antibacterial activity of rCqALF

Given that it is a type of antimicrobial peptide, the antimicrobial spectrum of rCqALF was determined in MIC and MBC assays. As shown in Table 2, rCqALF exhibited a broad spectrum of antimicrobial activity. Notably, rCqALF demonstrated antibacterial activity against both Gram-negative bacteria (*E. coli, P. fluorescens*, *S. flexneri* and *E. coli* MC1061) and Gram-positive bacteria (*M. luteus*, *C. glutamicum*, *B. subtilis* and *S. aureus*), which was similar to other

Table 2
Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of rCqALF.

Microorganisms	CGMCC No.[a]	rCqALF MIC[c] (μM)	rCqALF MBC[c] (μM)
Gram-negative bacteria (12)			
Pseudomonas stutzeri	CGMCC 1.1803	>44	>44
Edwardsiella tarda		>44	>44
Vibrio parahemolyticus	CGMCC 1.1615	>44	>44
Vibrio alginolyticus	CGMCC 1.1833	>44	>44
Vibrio harveyi	CGMCC 1.1593	>44	>44
Vibrio fluvialis	CGMCC 1.1609	>44	>44
Aeromonas hydrophila	CGMCC 1.0927	>44	>44
Aeromonas sobria		>44	>44
Escherichia coli	CGMCC 1.2389	22–44	22–44
Pseudomonas fluorescens	CGMCC 1.0032	22–44	44
Shigella flexneri	CGMCC 1.1868	2.75–5.5	2.75–5.5
Escherichia coli MC1061	ATCC: 25922[b]	2.75–5.5	2.75–5.5
Gram-positive bacteria (7)			
Micrococcus lysodeikticus Fleming	CGMCC 1.0634	>44	>44
Streptococcus agalactiae		>44	>44
Staphylococcus epidermidis	CGMCC 1.2429	>44	>44
Micrococcus luteus	CGMCC 1.634	5.5–11	5.5–11
Corynebacterium glutamicum	CGMCC 1.1886	22–44	22–44
Bacillus subtilis	CGMCC 1.447	22–44	22–44
Staphylococcus aureus	CGMCC 1.363	5.5–11	5.5–11
Yeast (1)			
Candida albicans	CGMCC 2.2411	>44	>44

[a] CGMCC No.: China General Microbiological Culture Collection Number.
[b] ATCC: American Type Culture Collection.
[c] MIC and MBC values indicate the minimum concentration of rCqALF required to inhibit 100% of bacterial growth and kill more than 99.9% of microorganisms, respectively.

AMPs in previous studies [38–40]. In addition, rCqALF showed strong activity against *S. flexneri* and *S. aureus*, which cause serious diseases in aquaculture, at a low bactericidal concentration (*S. flexneri*: MBC <6 μM and *S. aureus:* MBC < 12 μM). This result implies that the *CqALF* may efficiently protect red claw crayfish against bacterial infection, demonstrating that *CqALF* is a key immune-relevant molecule in crustaceans.

To assess the bactericidal effects of rCqALF on bacteria, a membrane integrity assay was performed with transgenic *E. coli* MC1061 to determine whether rCqALF affected bacterial membrane integrity. Strong light emission, an indication of membrane disruption, was observed after the addition of 1 μM cecropin P1, a well-known membrane-active antimicrobial peptide [41,42]. However, the presence of rCqALF (2.75 μM, 22 μM and 44 μM) only slightly increased the light intensity in comparison to the negative controls (25 μM argireline and MilliQ water treatments), although the difference was not significant (Fig. 9). This result indicated that rCqALF was unlikely to disrupt bacterial membrane integrity, in contrast to cecropin P1. The mechanism underlying the antibacterial activity of rCqALF still requires further investigation.

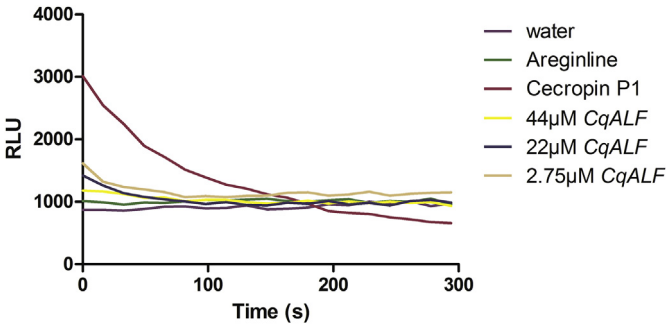

Fig. 9. The effects of rCqALF on bacterial membrane integrity. Light emission was determined following different treatments: MilliQ water, 25 μM argireline, 1 μM cecropin P1, 44 μM rCqALF, 22 μM rCqALF and 2.75 μM rCqALF. The related light units (RLUs) were determined over time.

4. Conclusion

In summary, this study highlights the role of *CqALF* in immunity given its antimicrobial activity and, in particular, its antiviral activity. The antiviral activity of rCqALF may be achieved via direct interaction between rCqALF and WSSV envelope proteins, which obviously disrupt WSSV envelope integrity and reduce WSSV replication in Hpt cell cultures *in vitro* and enhance protection against WSSV infection in animals *in vivo*. Further studies are required to elucidate how rCqALF interacts with the envelope protein(s) of WSSV and to determine which key amino acid(s) can be used for the design of novel antivirals. These studies will be helpful for WSSV disease control and the selection of feed additives in crustacean farming.

Acknowledgements

This work was supported by National Basic Research Program of China (973 program, 2012CB114403), the National Natural Science Foundation of China (No. 31222056, No. 41476117), MELRI1403, SFFPC (2013J06010) and FRFCU (20720150155).

References

[1] R.E. Hancock, G. Diamond, The role of cationic antimicrobial peptides in innate host defences, Trends Microbiol. 8 (2000) 402–410.
[2] V. Nizet, Antimicrobial peptide resistance mechanisms of human bacterial pathogens, Curr. Issues Mol. Biol. 8 (2006) 11–26.
[3] P. Jiravanichpaisal, S.Y. Lee, Y.A. Kim, T. Andren, I. Söderhäll, Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern, Dev. Comp. Immunol. 31 (2007) 441–455.
[4] A.Q. Yu, Y.H. Shi, Q. Wang, Characterisation of a novel Type I crustin involved in antibacterial and antifungal responses in the red claw crayfish, *Cherax quadricarinatus*, Fish Shellfish Immunol. 48 (2016) 30–38.
[5] S. Donpudsa, V. Rimphanitchayakit, A. Tassanakajon, I. Söderhäll, K. Söderhäll, Characterization of two crustin antimicrobial peptides from the freshwater crayfish *Pacifastacus leniusculus*, J. Invertebr. Pathol. 104 (2010) 234–238.
[6] H. Liu, P. Jiravanichpaisal, I. Söderhäll, L. Cerenius, K. Söderhäll, Antilipopolysaccharide factor interferes with white spot syndrome virus replication *in vitro* and *in vivo* in the crayfish *Pacifastacus leniusculus*, J. Virol. 80 (2006) 10365–10371.

[7] J. Aketagawa, T. Miyata, S. Ohtsubo, T. Nakamura, T. Morita, H. Hayashida, et al., Primary structure of limulus anticoagulant anti-lipopolysaccharide factor, J. Biol. Chem. 261 (1986) 7357–7365.
[8] T. Muta, T. Miyata, F. Tokunaga, T. Nakamura, S. Iwanaga, Primary structure of anti-lipopolysaccharide factor from American horseshoe crab, *Limulus polyphemus*, J. Biochem. 101 (1987) 1321–1330.
[9] P.S. Gross, T.C. Bartlett, C.L. Browdy, R.W. Chapman, G.W. Warr, Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*, Dev. Comp. Immunol. 25 (2001) 565–577.
[10] F. Liu, Y. Liu, F. Li, B. Dong, J. Xiang, Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp(*Fenneropenaeus chinensis*), Mar. Biotechnol. 7 (2005) 600–608.
[11] K.M. Beale, D.W. Towle, N. Jayasundara, C.M. Smith, J.D. Shields, H.J. Small, et al., Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: molecular characterization and transcriptional response to *Vibrio fluvialis* challenge, Comp. Biochem. Physiol. Part D Genom. Proteom. 3 (2008) 263–269.
[12] C. Imjongjirak, P. Amparyup, A. Tassanakajon, S. Sittipraneed, Anti-lipopolysaccharide factor (ALF) of mud crab *Scylla paramamosain*: molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain, Mol. Immunol. 44 (2007) 3195–3203.
[13] H.P. Liu, R.Y. Chen, Q.X. Zhang, Q.Y. Wang, C.R. Li, H. Peng, et al., Characterization of two isoforms of antiliopolysaccharide factors (Sp-ALFs) from the mud crab *Scylla paramamosain*, Fish Shellfish Immunol. 33 (2012) 1–10.
[14] H. Yang, S. Li, F. Li, J. Xiang, Structure and bioactivity of a modified peptide derived from the LPS-binding domain of an anti-lipopolysaccharide factor (ALF) of shrimp, Mar. Drugs (2016) 14.
[15] M. Krepstakies, J. Lucifora, C.H. Nagel, M.B. Zeisel, B. Holstermann, H. Hohenberg, et al., A new class of synthetic peptide inhibitors blocks attachment and entry of human pathogenic viruses, J. Infect. Dis. 205 (2012) 1654–1664.
[16] S. Suraprasit, T. Methatham, P. Jaree, K. Phiwsaiya, S. Senapin, I. Hirono, et al., Anti-lipopolysaccharide factor isoform 3 from *Penaeus monodon* (ALFPm3) exhibits antiviral activity by interacting with WSSV structural proteins, Antivir. Res. 110 (2014) 142–150.
[17] H.P. Liu, R.Y. Chen, Q.X. Zhang, H. Peng, K.J. Wang, Differential gene expression profile from haematopoietic tissue stem cells of red claw crayfish, *Cherax quadricarinatus*, in response to WSSV infection, Dev. Comp. Immunol. 35 (2011) 716–724.
[18] J. Jeswin, X.L. Xie, Q.L. Ji, K.J. Wang, H.P. Liu, Proteomic analysis by iTRAQ in red claw crayfish, *Cherax quadricarinatus*, hematopoietic tissue cells post white spot syndrome virus infection, Fish Shellfish Immunol. 50 (2016) 288–296.
[19] F.Y. Chen, H.P. Liu, J. Bo, H.L. Ren, K.J. Wang, Identification of genes differentially expressed in hemocytes of *Scylla paramamosain* in response to lipopolysaccharide, Fish Shellfish Immunol. 28 (2010) 167–177.
[20] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[21] J.L. Cereghino, J.M. Cregg, Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*, FEMS Microbiol. Rev. 24 (2000) 45–66.
[22] Q.X. Zhang, H.P. Liu, R.Y. Chen, K.L. Shen, K.J. Wang, Identification of a serine proteinase homolog (Sp-SPH) involved in immune defense in the mud crab *Scylla paramamosain*, PloS One 8 (2013) e63787.
[23] X. Xie, H. Li, L. Xu, F. Yang, A simple and efficient method for purification of intact white spot syndrome virus (WSSV) viral particles, Virus Res. 108 (2005) 63–67.
[24] L. Cai, J.J. Cai, H.P. Liu, D.Q. Fan, H. Peng, K.J. Wang, Recombinant medaka (*Oryzias melastigmus*) pro-hepcidin: multifunctional characterization, Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 161 (2012) 140–147.
[25] B. Chen, D.Q. Fan, K.X. Zhu, Z.G. Shan, F.Y. Chen, L. Hou, et al., Mechanism study on a new antimicrobial peptide Sphistin derived from the N-terminus of crab histone H2A identified in haemolymphs of *Scylla paramamosain*, Fish Shellfish Immunol. 47 (2015) 833–846.
[26] L. Cerenius, K. Söderhäll, The prophenoloxidase-activating system in invertebrates, Immunol. Rev. 198 (2004) 116–126.
[27] Y. Liu, Z. Cui, W. Luan, C. Song, Q. Nie, S. Wang, et al., Three isoforms of anti-lipopolysaccharide factor identified from eyestalk cDNA library of swimming crab *Portunus trituberculatus*, Fish Shellfish Immunol. 30 (2011) 583–591.
[28] R.D. Yedery, K.V. Reddy, Identification, cloning, characterization and recombinant expression of an anti-lipopolysaccharide factor from the hemocytes of Indian mud crab, *Scylla serrata*, Fish Shellfish Immunol. 27 (2009) 275–284.
[29] R. Keller, Crustacean neuropeptides: structures, functions and comparative aspects, Experientia 48 (1992) 439–448.
[30] E. de la Vega, N.A. O'Leary, J.E. Shockey, J. Robalino, C. Payne, C.L. Browdy, et al., Anti-lipopolysaccharide factor in *Litopenaeus vannamei* (LvALF): a broad spectrum antimicrobial peptide essential for shrimp immunity against bacterial and fungal infection, Mol. Immunol. 45 (2008) 1916–1925.
[31] S. Tharntada, S. Ponprateep, K. Somboonwiwat, H. Liu, I. Söderhäll, K. Söderhäll, et al., Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from white spot syndrome virus infection, J. Gen. Virol. 90 (2009) 1491–1498.
[32] J.H. Leu, F. Yang, X. Zhang, X. Xu, G.H. Kou, C.F. Lo, Whispovirus, Curr. Top. Microbiol. Immunol. 328 (2009) 197–227.
[33] H.-Y. Chou, M.-C. Tung, C.-F. Chang, M.-S. Su, G.-H. Kou, Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*, Dis. Aquat. Org. 23 (1995) 239–242.
[34] Y.S. Chang, W.J. Liu, C.C. Lee, T.L. Chou, Y.T. Lee, T.S. Wu, et al., A 3D model of the membrane protein complex formed by the white spot syndrome virus structural proteins, PloS One 5 (2010) e10718.
[35] P. Jiravanichpaisal, K. Söderhäll, I. Söderhäll, Characterization of white spot syndrome virus replication in *in vitro*-cultured haematopoietic stem cells of freshwater crayfish, *Pacifastacus leniusculus*, J. Gen. Virol. 87 (2006) 847–854.
[36] H. Yang, S. Li, F. Li, X. Lv, J. Xiang, Recombinant expression and functional analysis of an isoform of anti-lipopolysaccharide factors (FcALF5) from Chinese shrimp *Fenneropenaeus chinensis*, Dev. Comp. Immunol. 53 (2015) 47–54.
[37] K.C. Mulder, L.A. Lima, V.J. Miranda, S.C. Dias, O.L. Franco, Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides, Front. Microbiol. 4 (2013) 321.
[38] K. Krusong, P. Poolpipat, P. Supungul, A. Tassanakajon, A comparative study of antimicrobial properties of crustinPm1 and crustinPm7 from the black tiger shrimp *Penaeus monodon*, Dev. Comp. Immunol. 36 (2012) 208–215.
[39] B.J. Cuthbertson, L.J. Deterding, J.G. Williams, K.B. Tomer, K. Etienne, P.J. Blackshear, E.E. Büllesbach, P.S. Gross, Diversity in penaeidin antimicrobial peptide form and function, Dev. Comp. Immunol. 32 (2008) 167–181.
[40] F. Li, L. Wang, L. Qiu, H. Zhang, Y. Gai, L. Song, A double WAP domain-containing protein from Chinese mitten crab *Eriocheir sinensis* with antimicrobial activities against Gram-negative bacteria and yeast, Dev. Comp. Immunol. 36 (2012) 183–190.
[41] C. Li, H.M. Blencke, L.C. Smith, M.T. Karp, K. Stensvag, Two recombinant peptides, SpStrongylocins 1 and 2, from *Strongylocentrotus purpuratus*, show antimicrobial activity against Gram-positive and Gram-negative bacteria, Dev. Comp. Immunol. 34 (2010) 286–292.
[42] M. Virta, K.E. Akerman, P. Saviranta, C. Oker-Blom, M.T. Karp, Real-time measurement of cell permeabilization with low-molecular-weight membranolytic agents, J. Antimicrob. Chemother. 36 (1995) 303–315.