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A novel complement C3 like gene (*Lv-C3L*) from *Litopenaeus vannamei* with bacteriolytic and hemolytic activities and its role in antiviral immune response

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ABSTRACT

As a core component of the complement system, complement component 3 (C3) plays a central role in the opsonization of pathogens, immune defense and immune regulation in the mammalian for its activation is required to trigger classical as well as alternative complement pathways. However, the molecular mechanism underlying C3 activation in invertebrates remains unknown. Several C3 genes have been characterized in invertebrates but very few in crustacean. To understand the molecular characterization and immunological functions of shrimp C3, we characterized a novel complement C3 like gene (designated *Lv-C3L*) with full-length cDNA sequence identified from pacific white shrimp *Litopenaeus vannamei* in the present study. The full length cDNA of *Lv-C3L* sequence was 4769 bp (GenBank accession number: MH638255) containing a 4077 bp open reading frame (ORF), which encodes 1358 amino acids contained a putative signal peptide of 17 amino acids. Six model motifs of C3 were found in Lv-C3L including typical A2M domain, a highly conserved thioester region (GCGEQ) and proteolytic cleavage site of ANATO. In addition to typical conservative domains, Lv-C3L also contains a particular GLN-rich region which might be involved in the protein interaction and transcriptional activation. The transcripts of *Lv-C3L* were mainly detected in hemocytes and gill which might be involved in defense response. At 36 h post *V.parahaemolyticus* and *B.thuringensis* infection, the expression level of *Lv-C3L* gene in hemocytes were significantly upregulated. At 48 h and 72 h post WSSV infection, the expression level of *Lv-C3L* gene in hemocytes and gill were significantly upregulated. These results indicated that *Lv-C3L* gene play a pivotal role in innate immune responses to the WSSV and G^+/G^- bacterial infection. The obvious immune function of Lv-C3L was described as an effective membrane rupture in bacteriolytic and hemolytic activities on *V.parahaemolyticus*, *V.anguillarum* and rabbit erythrocytes. Combining with WSSV copy number, WSSV-*VP28* gene expression profile and shrimp cumulative mortality analysis, RNAi knockdown of *Lv-C3L* gene could obviously promote the *in vivo* propagation of WSSV in shrimp. This is the first report in crustaceans that Lv-C3L, as a key complement like components, is involved in shrimp antiviral immune response. It is speculated that complicated complement response cascade may exist in shrimp. These results collectively indicated that the complement pathway in shrimp might play an important protective role against pathogenic infection and activation of complement pathway including C3 could restrict the propagation of WSSV.

1. Introduction

Immunity to infection is mediated by two general systems: acquired (or adaptive) and innate (or natural). The innate immune system is an evolutionarily ancient form, and it is crucial for the first line of defense before the acquired immune system comes into play [1]. Innate immunity has considerable specificity and is capable of discriminating between pathogens and self, thereby successfully defending invertebrates and vertebrates against infection [2]. Complement could be activated by pathogenic surfaces through an antibody-independent pathway [3]. The complement system, which consists of three activation pathways including classic pathway, alternative pathway and the lectin pathway, is engaged in both acquired and innate immunity [4–6]. The classical pathway is activated by antibody-antigen complexes and is a major effector of antibody-

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mediated immunity. The other two pathways (lectin and alternative) function in innate immune defense. The complement system is a cascade system composed of 40 factors in plasma or on cytoplasmic membrane that are regulated through the precise mechanism. These factors belongs to four types, including inherent components, regulatory molecules, complement receptors and activation of specific protein fragments [7]. The complement system plays important immune surveillance role in humoral immunity, and provides a connection between innate immunity and adaptive immunity. As a member of many immune mechanisms, the complement system functions at the end of the immune process of the effective stage [8].

Complement component 3 (C3), was first identified in 1912 [9]. Relative to other complement components, C3 is present in serum with the highest proportion. C3 is a β2 glycoprotein containing two peptide chains (α and β) and belongs to the thioester-containing protein (TEP) superfamily [10]. C3 is continuously activated at a low rate in the fluid phase through either cleavage by serum proteases to C3a and C3b, reaction of the thioester with small nucleophiles or water, or non-specific perturbations leading to conformational changes and the exposure and hydrolysis of the thioester [11]. Activated C3 regulates the complement system, transferring the signal to C5 or the terminal pathway. The activated fragments of C3 (C3a and C3b) are involved as a factor that activates macrophages and mastocytes [12]. C3 is the core, the most abundant and complex factor in various components molecule of complement system as it is the intersection of several activation pathways, and depends on a positive feedback loop of C3b. The C3 gene is conserved and exists in vertebrates and many invertebrate species [13]. Complement C3 is activated via three convergent pathways (classical, alternative, and lectin). The activation of C3 can lead to a variety of host-defense systems, including the promotion of phagocytosis and the formation of a membrane-attack complex [14].

To date, the C3 gene has been reported in many vertebrates and invertebrate species [13]. In vertebrates, there have been much researches on C3 expounded the structure, biological activity and functional properties. C3 isoforms have been found in marine invertebrates, such as *Carcinoscorpius rotundicauda* [15], *Lottia gigantea* [16], *Crassostrea gigas* [17] and *Sinonovacula constricta* [18]. Most of them focus on basic descriptions, expression patterns and characterizations from transcriptome and genome. However, few studies have characterized C3 and C3 activated fragments. Research on the function and activation pathway of C3 in invertebrates is still not clear. The culture production of *Litopenaeus vannamei* (*L. vannamei*) accounts for about 85% of the total shrimp production in mainland of China [19]. Both virus and bacteria can be dangerous pathogens for healthy development of shrimp in aquaculture. In the last decade, researches focused on shrimp immunity were published and a batch of related data accumulated. Several functional genes, involved in immune recognition, humoral or cellular immune response were reported [19–23]. As an important part of the innate immune system, C3 is particularly crucial to determine the health status of invertebrate because they lack adaptive immune systems. Although C3 and C3 isoforms have been studied in some marine invertebrate, there are still no related reports on shrimp C3 or complement system. In this research, the expression characteristics and the role in innate immune responses of the C3 like gene in *L. vannamei* were analyzed. This study would help increase understanding the function of C3 gene in invertebrate immune system and give new evidence for further research on the primary complement molecules in invertebrates.

2. Materials and methods

2.1. Experimental animal and tissue collection

Healthy *L. vannamei* (average body weight 20.0 ± 3 g and body length of 14 ± 2 cm) were collected from Chaolin Aquaculture Co., Ltd., Xiqing district, Tianjin city, China. They were cultured in a recirculating water tank system filled with air-pumped sea water with 3% salinity at 26 °C, fed with commercial diets for 7 days and acclimated to the laboratory condition.

For tissue distribution analysis, hemolymph of 10 healthy adult shrimps were obtained by using a sterile syringe preloaded with equal volume of modified anticoagulant Alsever solution and then centrifuged immediately at 800 g at 4 °C for 10 min [24]. After centrifugation, the hemocytes were collected and preserved in TRIzol Reagent (Invitrogen, USA) for RNA isolation. Then, the hepatopancreas, heart, muscle, intestine, stomach, gill, nerve and pleoplod were dissected and kept in liquid nitrogen for RNA isolation.

2.2. Pathogens and immune challenge

Vibrio parahaemolyticus and *Bacillus thuringensis* were cultured in Trypticase Soy Broth (TSB) medium at 30 °C and Nutrient Broth (NB) medium at 37 °C, respectively. Then, the concentration of bacterial suspension was adjusted to 1×10^7 CFU/mL with sterile Phosphate Buffered Saline (PBS) and was measured by SmartSpec™ Plus Spectrophotometer (BIO-RAD, USA). Each shrimp in bacterial challenge group was injected with 50 μL above bacterial suspension. That in control group was injected with 50 μL sterilized PBS. Dead and moribund WSSV-infected crayfishes (*Procambarus clarkia*) were collected and kept at 4 °C for virus purification. Gill tissue was homogenized and prepared as WSSV inoculum to a final concentration (1×10^5 virions/10 μL) in TN buffer (20 mM Tris-HCl, 400 mM NaCl, pH 7.4) following published methods [25]. A qRT-PCR method was used for detecting the number of copies about the WSSV. For the WSSV challenge experiment, each experimental and control animal were injected with 10 μL WSSV inoculum and sterile TN buffer, respectively. 70 healthy shrimps were used in experimental and control group. Hemocytes and gill of seven shrimps in each group were collected for real time RT-PCR at 6, 12, 24, 36, 48, 60 and 72 (h) post bacterial infection (hpi) and at 0.5, 5, 12, 24, 48 and 72 (h) post WSSV infection (hpi).

2.3. Total RNA extraction and cDNA synthesis

Total RNA of each tissue was extracted by TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA concentration was assessed by Nanodrop 2000 (Thermo Fisher Scientific, USA) and the RNA quality was assessed by electrophoresis on 1% agarose gel. All cDNA samples were synthesized from 2 μg total RNA with GoScript™ Reverse Transcription System (Promega, USA). The first-strand cDNA was synthesized by GoScript™ Reverse Transcriptase following the manufacturer's instructions with Oligo $(dT)_{16}$ primer (AOLP) and adapter primer (BDA) (Table 1).

2.4. Cloning of the full length cDNA sequence of Lv-C3L

The partial sequence of Lv-C3L cDNA was obtained from an Illumina-based transcriptome sequencing database of *L. vannamei* hemocytes built in our lab [26]. Primers Lv-C3L F1/Lv-C3L R1 and Lv-C3L F2/Lv-C3L R2 (Table 1) were designed to validate the sequence. Based on the partial sequence of *Lv-C3L*, the 3′ and 5′ ends were obtained by RACE PCR approaches, using gene specific primers and anchor primers. Four specific primers (Lv-C3L 3F1, Lv-C3L 3F2, Lv-C3L 5R1, Lv-C3L 5R2) and two anchor primers AP and NUP (Table 1) were designed for direct and semi-nested PCR. The PCR program for the amplification of *Lv-C3L* was as follows: 1 cycle of denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55–60 °C for 1 min, extension at 72 °C for 1 min, followed by an extension at 72 °C for 10 min. The specific products were assessed by electrophoresis on 1% agarose gel. Then, the amplified products were purified using SanPrep Column DNA Gel Extraction Kit (Sangon, China) and cloned into pMD19-T vector (Takara, Beijing) and then transformed into DH5α competent cell for sequencing at Life Technologies (China).

Table 1
Information of primer sequences used in the present study.

Primers name	Sequence (5′-3′)	Comments
BDA	AAGCAGTGGTATCAACGCAGAAGTACGCGGG	adaptor primer
AOLP	GGCCACGCGTCGACTAGTAC$(T)_{16}$(A/C/G)	Oligo$(dT)_{16}$ primer
Lv-C3L F1	CGTTTACGGATGGAAG	primer for fragment amplification of *Lv-C3L*
Lv-C3L R1	CGACACGGTGAGATTG	primer for fragment amplification of *Lv-C3L*
Lv-C3L F2	AGGAGTCGGGAAGCGTAA	primer for fragment amplification of *Lv-C3L*
Lv-C3L R2	TCGGGCTGGTAGTAGTCGTA	primer for fragment amplification of *Lv-C3L*
NUP	AAGCAGTGGTATCAACGCAGAGT	adaptor primer
Lv-C3L 5R1	GAGTGCGAGTTCTGGAGGTTTATG	5′ RACE primer of *Lv-C3L*
Lv-C3L 5R2	GAAGTCGTAGCACTGGTGGGATTG	5′ RACE primer of *Lv-C3L*
AP	GGCCACGCGTCGACTAGTAC	reverse transcription primer
Lv-C3L 3F1	AGCCTGTGCTGCCTACCT	3′ RACE primer of *Lv-C3L*
Lv-C3L 3F2	CGATGTGCGATGGTTTCA	3′ RACE primer of *Lv-C3L*
Lv-C3L qF	GAAGGAACAGCCAGGAGAGAA	qPCR primer of *Lv-C3L*
Lv-C3L qR	GGTAGATTTTGTAGTCACCCAGG	qPCR primer of *Lv-C3L*
TPI-qF	GGTCACGTGTTGTCTTGGCTTATGAACC	qPCR primer of internal reference gene for pathogenic infection
TPI-qR	CTGAACAAAGTCTGGCTTGAGAGCTGC	qPCR primer of internal reference gene for pathogenic infection
β-actin-F	GAAGTAGCCGCCCTGGTTG	qPCR primer of internal reference gene for tissue distribution
β-actin-R	CGGTTAGCCTTGGGGTTGAGGGGAG	qPCR primer of internal reference gene for tissue distribution
Lv-C3L EF	GGAATTCCATATGCTGCAGATCCAAGTGCC	primer for prokaryotic expression of *Lv-C3L*
Lv-C3L ER	CCCAAGCTTTCAGTGGTGGTGGTGGTGGTGAAGGTGGACCGTCACCT	primer for prokaryotic expression of *Lv-C3L*
Lv-C3L-dsF	ACATGGCTGTGGGACATCGTT	primer of *Lv-C3L* dsRNA
Lv-C3L-dsR	AAGGTGGACCGTCACCTTCA	primer of *Lv-C3L* dsRNA
T7-Lv-C3L-dsF	GATCACTAATACGACTCACTATAGGGACATGGCTGTGGGACATCGTT	primer of *Lv-C3L* dsRNA with T7 promoter
T7-Lv-C3L-dsR	GATCACTAATACGACTCACTATAGGGAAGGTGGACCGTCACCTTCA	primer of *Lv-C3L* dsRNA with T7 promoter
EGFP-dsF	CAGTGCTTCAGCCGCTACCC	primer of *EGFP* dsRNA
EGFP-dsR	TTCACCTTGATGCCGTTCTT	primer of *EGFP* dsRNA
T7-EGFP-dsF	GATCACTAATACGACTCACTATAGGGCAGTGCTTCAGCCGCTACCC	primer of *EGFP* dsRNA with T7 promoter
T7-EGFP-dsR	GATCACTAATACGACTCACTATAGGGTTCACCTTGATGCCGTTCTT	primer of *EGFP* dsRNA with T7 promoter
VP28-qF	GGGAACATTCAAGGTGTGGA	qPCR primer of WSSV-*VP28*
VP28-qR	GGTGAAGGAGGAGGTGTTGG	qPCR primer of WSSV-*VP28*
W71F	CTGACGACCTCTTCAAAT	qPCR primer of WSSV genome
W71R	TTCGCTATCTTCATAATC	qPCR primer of WSSV genome

2.5. Sequence and phylogenetic analysis

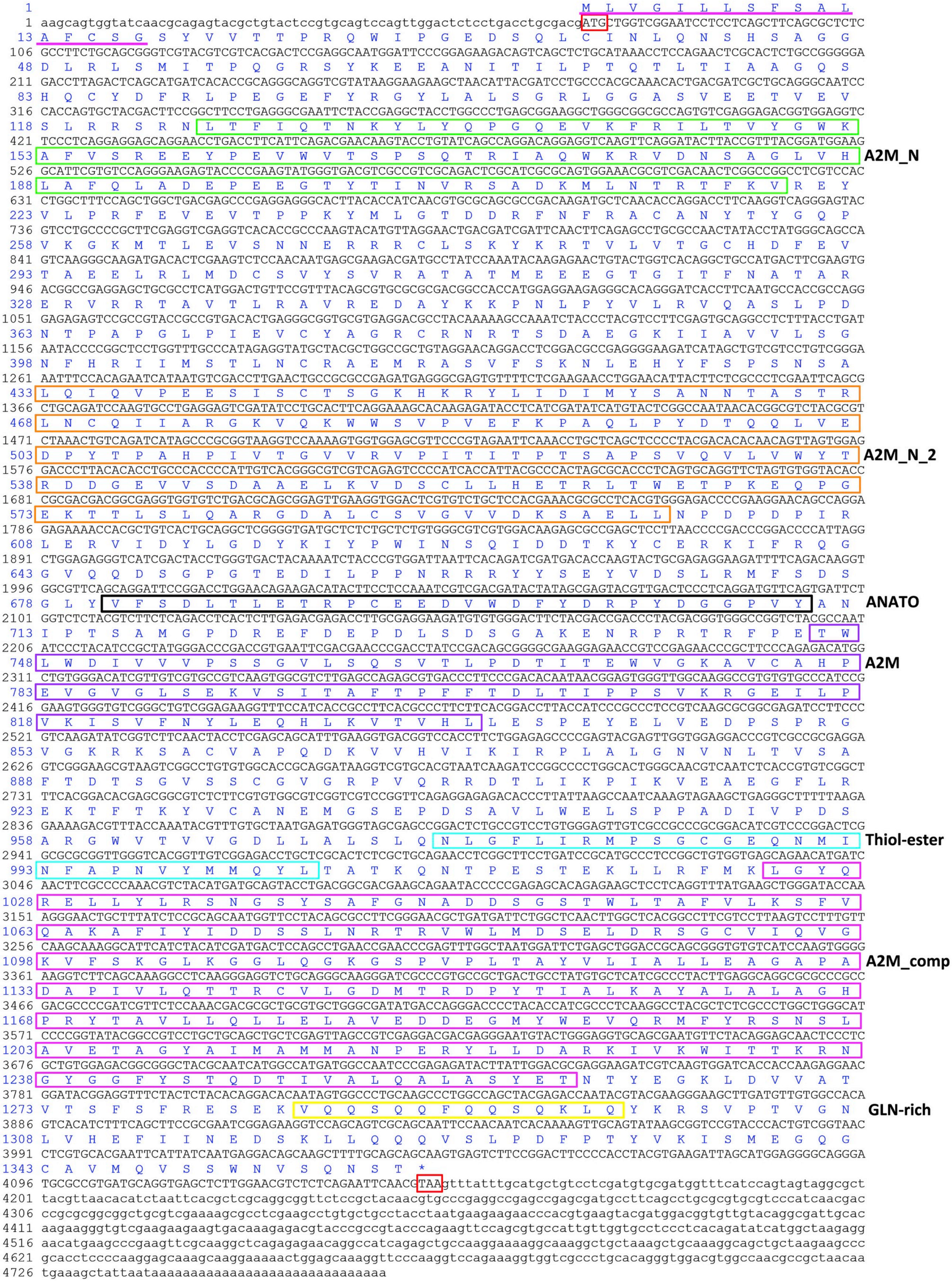
The complete ORF region and amino acid sequence of *Lv-C3L* was deduced using the translate tool in ExPASy (http://web.expasy.org/translate/). The molecular weight and isoelectric point were predicted using Compute pI/Mw tool (http://web.expasy.org/compute.pi/). The ORF sequence and deduced protein sequence of *Lv-C3L* were analyzed with BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/). SignalP 4.1 Server tool (http://www.cbs.dtu.dk/Services/Signal P/) was applied to predict Signal peptide cleavage sites. The TMHMM Server v.2.0 tool (http://www.cbs.dtu.dk/services/TMHMM-2.0/) was used to predict the transmembrane helices in Lv-C3L. The number and position of potential N-glycosylation sites were predicted using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc). Sixteen protein sequences of C3 family members from different species were used to perform multiple alignments using Clustal X and Bioedit. A phylogenic tree was constructed by the neighbor-joining (NJ) algorithm using the MEGA 5 software. The reliability of the tree was tested by bootstrapping using 1,000 replications.

2.6. Quantitative real-time PCR detection of Lv-C3L mRNA expression

SYBR Green-based quantitative real-time PCR (qRT-PCR) was performed to detect the gene expression level of Lv-C3L by using 7500 Fast Real-time PCR system (Applied Biosystems, USA). A pair of primers Lv-C3L qF and Lv-C3L qR (Table 1) was designed to detect the expression level of *Lv-C3L* in different tissues and samples after bacterial or WSSV infection. Primers β-actin (F/R) and TPI (F/R) (Table 1) were designed to detect the expression of the internal reference gene, β-actin and triosephosphate isomerase. All samples were repeated in triplicate when analyzed by real-time PCR ($n = 3$). The PCR products of these two expected bands were firstly sequenced to confirm the specificity and effectiveness of primers for real-time PCR. The program was as follows: denaturation at 95 °C for 5 min; 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The PCR product was denatured to produce melting curve to check the specificity of the PCR product. The data obtained from the real-time PCR analysis were analyzed using the comparative CT method according to the user manual. Statistical analysis was performed with SPSS software (version 19.0). The data were analyzed with a one-way ANOVA followed by Duncan and Tukey multiple comparison tests. Differences were considered to be significant at $P < 0.05$.

2.7. Expression and purification of recombinant protein (rLv-C3L), antiserum preparation and Ab purification

For the expression of mutated protein, conventional methods were used to construct the recombinant vector by using restriction endonuclease *Nde* I and *Hind* III. The DNA fragments encoding A2M domain of Lv-C3L was amplified by primers (Lv-C3L EF and Lv-C3L ER) listed in Table 1. The amplification product was cloned into expression vector pET-28a(+) (Novagen). BL21(DE3)pLysS competent cells were transformed by the recombinant plasmid, and then fermented in LB medium containing 100 mg/mL ampicillin at 37 °C until OD_{600} reached 0.6 (about 10^6 *E. coli*/mL medium). Isopropyl β-D-thiogalactopyranoside (IPTG) was added into medium for inducing expression of rLv-C3L at 37 °C for 6 h. The His-tagged rLv-C3L was purified through Immobilized Mental-Affinity Chromatography (IMAC) column by using ÄKTA™ prime Plus (GE). Purification, refolding and identification of the recombinant protein by LC-ESI-MS were according to the procedure in our lab [27]. Antibody of Lv-C3L was generated in New Zealand white rabbit and purified with Protein A resin column (GE) by a commercial company (Beijing Protein Innovation Co., China). The eluted Abs were neutralized immediately with 1/10 volume of 1 M Tris-HCl (pH 8.5)



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   1                                                                          M  L  V  G  I  L  L  S  F  S  A  L
   1 aagcagtggtatcaacgcagagtacgctgtactccgtgcagtccagttggactctcctgacctgcgacgATGCTGGTCGGAATCCTCCTCAGCTTCAGCGCTCTC
  13  A  F  C  S  G  S  Y  V  V  T  T  P  R  Q  W  I  P  G  E  D  S  Q  L  C  I  N  L  Q  N  S  H  S  A  G  G
 106 GCCTTCTGCAGCGGGTCGTACGTCGTCACGACTCCGAGGCAATGGATTCCCGGAGAAGACAGTCAGCTCTGCATAAACCTCCAGAACTCGCACTCTGCCGGGGGA
  48  D  L  R  L  S  M  I  T  P  Q  G  R  S  Y  K  E  E  A  N  I  T  I  L  P  T  Q  T  L  T  I  A  A  G  Q  S
 211 GACCTTAGACTCAGCATGATCACACCGCAGGGCAGGTCGTATAAGGAAGAAGCTAACATTACGATCCTGCCCACGCAAACACTGACGATCGCTGCAGGGCAATCC
  83  H  Q  C  Y  D  F  R  L  P  E  G  E  F  Y  R  G  Y  L  A  L  S  G  R  L  G  G  A  S  V  E  E  T  V  E  V
 316 CACCAGTGCTACGACTTCCGGCTTCCTGAGGGCGAATTCTACCGAGGCTACCTGGCCCTGAGCGGAAGGCTGGGCGGCGCCAGTGTCGAGGAGACGGTGGAGGTC
 118  S  L  R  R  S  R  N  L  T  F  I  Q  T  N  K  Y  L  Y  Q  P  G  Q  E  V  K  F  R  I  L  T  V  Y  G  W  K
 421 TCCCTCAGGAGGAGCAGGAACCTGACCTTCATTCAGACGAACAAGTACCTGTATCAGCCAGGACAGGAGGTCAAGTTCAGGATACTTACCGTTTACGGATGGAAG
 153  A  F  V  S  R  E  E  Y  P  E  V  W  V  T  S  P  S  Q  T  R  I  A  Q  W  K  R  V  D  N  S  A  G  L  V  H  A2M_N
 526 GCATTCGTGTCCAGGGAAGAGTACCCCGAAGTATGGGTGACGTCGCCGTCGCAGACTCGCATCGCGCAGTGGAAACGCGTCGACAACTCGGCCGGCCTCGTCCAC
 188  L  A  F  Q  L  A  D  E  P  E  E  G  T  Y  T  I  N  V  R  S  A  D  K  M  L  N  T  R  T  F  K  V  R  E  Y
 631 CTGGCTTTCCAGCTGGCTGACGAGCCCGAGGAGGGCACTTACACCATCAACGTGCGCAGCGCCGACAAGATGCTCAACACCAGGACCTTCAAGGTCAGGGAGTAC
 223  V  L  P  R  F  E  V  E  V  T  P  P  K  Y  M  L  G  T  D  D  R  F  N  F  R  A  C  A  N  Y  T  Y  G  Q  P
 736 GTCCTGCCCCGCTTCGAGGTCGAGGTCACACCGCCCAAGTACATGTTAGGAACTGACGATCGATTCAACTTCAGAGCCTGCGCCAACTATACCTATGGGCAGCCA
 258  V  K  G  K  M  T  L  E  V  S  N  N  E  R  R  R  C  L  S  K  Y  K  R  T  V  L  V  T  G  C  H  D  F  E  V
 841 GTCAAGGGCAAGATGACACTCGAAGTCTCCAACAATGAGCGAAGACGATGCCTATCCAAATACAAGAGAACTGTACTGGTCACAGGCTGCCATGACTTCGAAGTG
 293  T  A  E  E  L  R  L  M  D  C  S  V  Y  S  V  R  A  T  A  T  M  E  E  E  G  T  G  I  T  F  N  A  T  A  R
 946 ACGGCCGAGGAGCTGCGCCTCATGGACTGTTCCGTTTACAGCGTGCGCGCGACGGCCACCATGGAGGAAGAGGGCACAGGGATCACCTTCAATGCCACCGCCAGG
 328  E  R  V  R  R  T  A  V  T  L  R  A  V  R  E  D  A  Y  K  K  P  N  L  P  Y  V  L  R  V  Q  A  S  L  P  D
1051 GAGAGAGTCCGCCGTACCGCCGTGACACTGAGGGCGGTGCGTGAGGACGCCTACAAAAAGCCAAATCTACCCTACGTCCTTCGAGTGCAGGCCTCTTTACCTGAT
 363  N  T  P  A  P  G  L  P  I  E  V  C  Y  A  G  R  C  R  N  R  T  S  D  A  E  G  K  I  I  A  V  V  L  S  G
1156 AATACCCCGGCTCCTGGTTTGCCCATAGAGGTATGCTACGCTGGCCGCTGTAGGAACAGGACCTCGGACGCCGAGGGGAAGATCATAGCTGTCGTCCTGTCGGGA
 398  N  F  H  R  I  I  M  S  T  L  N  C  R  A  E  M  R  A  S  V  F  S  K  N  L  E  H  Y  F  S  P  S  N  S  A
1261 AATTTCCACAGAATCATAATGTCGACCTTGAACTGCCGCGCCGAGATGAGGGCGAGTGTTTTCTCGAAGAACCTGGAACATTACTTCTCGCCCTCGAATTCAGCG
 433  L  Q  I  Q  V  P  E  E  S  I  S  C  T  S  G  K  H  K  R  Y  L  I  D  I  M  Y  S  A  N  N  T  A  S  T  R
1366 CTGCAGATCCAAGTGCCTGAGGAGTCGATATCCTGCACTTCAGGAAAGCACAAGAGATACCTCATCGATATCATGTACTCGGCCAATAACACGGCGTCTACGCGT
 468  L  N  C  Q  I  I  A  R  G  K  V  Q  K  W  W  S  V  P  V  E  F  K  P  A  Q  L  P  Y  D  T  Q  Q  L  V  E
1471 CTAAACTGTCAGATCATAGCCCGCGGTAAGGTCCAAAAGTGGTGGAGCGTTCCCGTAGAATTCAAACCTGCTCAGCTCCCCTACGACACACAACAGTTAGTGGAG
 503  D  P  Y  T  P  A  H  P  I  V  T  G  V  V  R  V  P  I  T  I  T  P  T  S  A  P  S  V  Q  V  L  V  W  Y  T  A2M_N_2
1576 GACCCTTACACACCTGCCCACCCCATTGTCACGGGCGTCGTCAGAGTCCCCATCACCATTACGCCCACTAGCGCACCCTCAGTGCAGGTTCTAGTGTGGTACACC
 538  R  D  D  G  E  V  V  S  D  A  A  E  L  K  V  D  S  C  L  L  H  E  T  R  L  T  W  E  T  P  K  E  Q  P  G
1681 CGCGACGACGGCGAGGTGGTGTCTGACGCAGCGGAGTTGAAGGTGGACTCGTGTCTGCTCCACGAAACGCGCCTCACGTGGGAGACCCCGAAGGAACAGCCAGGA
 573  E  K  T  T  L  S  L  Q  A  R  G  D  A  L  C  S  V  G  V  V  D  K  S  A  E  L  L  N  P  D  P  D  P  I  R
1786 GAGAAAACCACGCTGTCACTGCAGGCTCGGGGTGATGCTCTCTGCTCTGTGGGCGTCGTGGACAAGAGCGCCGAGCTCCTTAACCCCGACCCGGACCCCATTAGG
 608  L  E  R  V  I  D  Y  L  G  D  Y  K  I  Y  P  W  I  N  S  Q  I  D  D  T  K  Y  C  E  R  K  I  F  R  Q  G
1891 CTGGAGAGGGTCATCGACTACCTGGGTGACTACAAAATCTACCCGTGGATTAATTCACAGATCGATGACACCAAGTACTGCGAGAGGAAGATTTTCAGACAAGGT
 643  G  V  Q  Q  D  S  G  P  G  T  E  D  I  L  P  P  N  R  R  R  Y  Y  S  E  Y  V  D  S  L  R  M  F  S  D  S
1996 GGCGTTCAGCAGGATTCCGGACCTGGAACAGAAGACATACTTCCTCCAAATCGTCGACGATACTATAGCGAGTACGTTGACTCCCTCAGGATGTTCAGTGATTCT
 678  G  L  Y  V  F  S  D  L  T  L  E  T  R  P  C  E  E  D  V  W  D  F  Y  D  R  P  Y  D  G  G  P  V  Y  A  N  ANATO
2101 GGTCTCTACGTCTTCTCAGACCTCACTCTTGAGACGAGACCTTGCGAGGAAGATGTGTGGGACTTCTACGACCGACCCTACGACGGTGGGCCGGTCTACGCCAAT
 713  I  P  T  S  A  M  G  P  D  R  E  F  D  E  P  D  L  S  D  S  G  A  K  E  N  R  P  R  T  R  F  P  E  T  W
2206 ATCCCTACATCCGCTATGGGACCCGACCGTGAATTCGACGAACCCGACCTATCCGACAGCGGGGCGAAGGAGAACCGTCCGAGAACCCGCTTCCCAGAGACATGG
 748  L  W  D  I  V  V  V  P  S  S  G  V  L  S  Q  S  V  T  L  P  D  T  I  T  E  W  V  G  K  A  V  C  A  H  P  A2M
2311 CTGTGGGACATCGTTGTCGTGCCGTCAAGTGGCGTCTTGAGCCAGAGCGTGACCCTTCCCGACACAATAACGGAGTGGGTTGGCAAGGCCGTGTGTGCCCATCCG
 783  E  V  G  V  G  L  S  E  K  V  S  I  T  A  F  T  P  F  F  T  D  L  T  I  P  P  S  V  K  R  G  E  I  L  P
2416 GAAGTGGGTGTCGGGCTGTCGGAGAAGGTTTCCATCACCGCCTTCACGCCCTTCTTCACGGACCTTACCATCCCGCCCTCCGTCAAGCGCGGCGAGATCCTTCCC
 818  V  K  I  S  V  F  N  Y  L  E  Q  H  L  K  V  T  V  H  L  L  E  S  P  E  Y  E  L  V  E  D  P  S  P  R  G
2521 GTCAAGATATCGGTCTTCAACTACCTCGAGCAGCATTTGAAGGTGACGGTCCACCTTCTGGAGAGCCCCGAGTACGAGTTGGTGGAGGACCCGTCGCCGCGAGGA
 853  V  G  K  R  K  S  A  C  V  A  P  Q  D  K  V  V  H  V  I  K  I  R  P  L  A  L  G  N  V  N  L  T  V  S  A
2626 GTCGGGAAGCGTAAGTCGGCCTGTGTGGCACCGCAGGATAAGGTCGTGCACGTAATCAAGATCCGGCCCCTGGCACTGGGCAACGTCAATCTCACCGTGTCGGCT
 888  F  T  D  T  S  G  V  S  S  C  G  V  G  R  P  V  Q  R  R  D  T  L  I  K  P  I  K  V  E  A  E  G  F  L  R
2731 TTCACGGACACGAGCGGCGTCTCTTCGTGTGGCGTCGGTCGTCCGGTTCAGAGGAGAGACACCCTTATTAAGCCAATCAAAGTAGAAGCTGAGGGCTTTTTAAGA
 923  E  K  T  F  T  K  Y  V  C  A  N  E  M  G  S  E  P  D  S  A  V  L  W  E  L  S  P  P  A  D  I  V  P  D  S
2836 GAAAAGACGTTTACCAAATACGTTTGTGCTAATGAGATGGGTAGCGAGCCGGACTCTGCCGTCCTGTGGGAGTTGTCGCCGCCCGCGGACATCGTCCCGGACTCG
 958  A  R  G  W  V  T  V  V  G  D  L  L  A  L  S  L  Q  N  L  G  F  L  I  R  M  P  S  G  C  G  E  Q  N  M  I  Thiol-ester
2941 GCGCGCGGTTGGGTCACGGTTGTCGGAGACCTGCTCGCACTCTCGCTGCAGAACCTCGGCTTCCTGATCCGCATGCCCTCCGGCTGTGGTGAGCAGAACATGATC
 993  N  F  A  P  N  V  Y  M  M  Q  Y  L  T  A  T  K  Q  N  T  P  E  S  T  E  K  L  L  R  F  M  K  L  G  Y  Q
3046 AACTTCGCCCCAAACGTCTACATGATGCAGTACCTGACGGCGACGAAGCAGAATACCCCCGAGAGCACAGAGAAGCTCCTCAGGTTTATGAAGCTGGGATACCAA
1028  R  E  L  L  Y  L  R  S  N  G  S  Y  S  A  F  G  N  A  D  D  S  G  S  T  W  L  T  A  F  V  L  K  S  F  V
3151 AGGGAACTGCTTTATCTCCGCAGCAATGGTTCCTACAGCGCCTTCGGGAACGCTGATGATTCTGGCTCAACTTGGCTCACGGCCTTCGTCCTTAAGTCCTTTGTT
1063  Q  A  K  A  F  I  Y  I  D  D  S  S  L  N  R  T  R  V  W  L  M  D  S  E  L  D  R  S  G  C  V  I  Q  V  G
3256 CAAGCAAAGGCATTCATCTACATCGATGACTCCAGCCTGAACCGAACCCGAGTTTGGCTAATGGATTCTGAGCTGGACCGCAGCGGGTGTGTCATCCAAGTGGGG
1098  K  V  F  S  K  G  L  K  G  G  L  Q  G  K  G  S  P  V  P  L  T  A  Y  V  L  I  A  L  L  E  A  G  A  P  A  A2M_comp
3361 AAGGTCTTCAGCAAAGGCCTCAAGGGAGGTCTGCAGGGCAAGGGATCGCCCGTGCCGCTGACTGCCTATGTGCTCATCGCCCTACTTGAGGCAGGCGCGCCCGCC
1133  D  A  P  I  V  L  Q  T  T  R  C  V  L  G  D  M  T  R  D  P  Y  T  I  A  L  K  A  Y  A  L  A  L  A  G  H
3466 GACGCCCCGATCGTTCTCCAAACGACGCGCTGCGTGCTGGGCGATATGACCAGGGACCCCTACACCATCGCCCTCAAGGCCTACGCTCTCGCCCTGGCTGGGCAT
1168  P  R  Y  T  A  V  L  L  Q  L  L  E  L  A  V  E  D  D  E  G  M  Y  W  E  V  Q  R  M  F  Y  R  S  N  S  L
3571 CCCCGGTATACGGCCGTCCTGCTGCAGCTGCTCGAGTTAGCCGTCGAGGACGACGAGGGAATGTACTGGGAGGTGCAGCGAATGTTCTACAGGAGCAACTCCCTC
1203  A  V  E  T  A  G  Y  A  I  M  A  M  M  A  N  P  E  R  Y  L  L  D  A  R  K  I  V  K  W  I  T  T  K  R  N
3676 GCTGTGGAGACGGCGGGCTACGCAATCATGGCCATGATGGCCAATCCCGAGAGATACTTATTGGACGCGAGGAAGATCGTCAAGTGGATCACCACCAAGAGGAAC
1238  G  Y  G  G  F  Y  S  T  Q  D  T  I  V  A  L  Q  A  L  A  S  Y  E  T  N  T  Y  E  G  K  L  D  V  V  A  T
3781 GGATACGGAGGTTTCTACTCTACACAGGACACAATAGTGGCCCTGCAAGCCCTGGCCAGCTACGAGACCAATACGTACGAAGGGAAGCTTGATGTTGTGGCCACA
1273  V  T  S  F  S  F  R  E  S  E  K  V  Q  Q  S  Q  Q  F  Q  Q  S  Q  K  L  Q  Y  K  R  S  V  P  T  V  G  N  GLN-rich
3886 GTCACATCTTTCAGCTTCCGCGAATCGGAGAAGGTCCAGCAGTCGCAGCAATTCCAACAATCACAAAAGTTGCAGTATAAGCGGTCCGTACCCACTGTCGGTAAC
1308  L  V  H  E  F  I  I  N  E  D  S  K  L  L  Q  Q  Q  V  S  L  P  D  F  P  T  Y  V  K  I  S  M  E  G  Q  G
3991 CTCGTGCACGAATTCATTATCAATGAGGACAGCAAGCTTTTGCAGCAGCAAGTGAGTCTTCCGGACTTCCCCACCTACGTGAAGATTAGCATGGAGGGGCAGGGA
1343  C  A  V  M  Q  V  S  S  W  N  V  S  Q  N  S  T  *
4096 TGCGCCGTGATGCAGGTGAGCTCTTGGAACGTCTCTCAGAATTCAACGTAAgtttatttgcatgctgtcctcgatgtgcgatggtttcatccagtagtaggcgct
4201 tacgttaacacatctaattcacgctcgcaggcggttctccgctacaacgtgcccgaggccgagccgagcgatgccttcagcctgcgcgtgcgtcccatcaacgac
4306 ccgcgcggcggctgcgtcgaaaagcgcctcgaagcctgtgctgcctacctaatgaagaagaacccacgtgaagtacgatggacggtgttgtacaggcgattgcac
4411 aagaagggtgtcgaagaagaagtgacaaagagacgtacccgccgtacccagaagttccagcgtgccattgttggtgcctccctcacagatatcatggctaagagg
4516 aacatgaagcccgaagttcgcaaggctcagagagaacaggccatcagagctgccaaggaaaaggcaaaggctgctaaagctgcaaaggcagctgctaagaagccc
4621 gcacctccccaaggagcaaagcaaggaaaaactggagcaaaggttcccaaggtccagaaaggtggtcgccctgcacagggtggacgtggccaacgccgctaacaa
4726 tgaaagctattaataaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Fig. 1. The complete nucleotide and deduced amino acid sequence of Lv-C3L from *L. vannamei*. The letter in red box indicates the start codon (ATG) and the stop codon (TAA). The putative sequence of signal peptide is underlined with pink single line. Seven functional domains and motifs are shown in green box (A2M_N domain), orange box (A2M_N_2 domain), black box (ANATO domain), purple box (A2M domain), blue box (Thiol-ester region), pink box (A2M_comp domain) and yellow box (GLN-rich region), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and then dialyzed in PBS overnight at 4 °C.

2.8. Preparation of *L. vannamei*'s plasma (LVP) and western blotting analysis

LVP was obtained from the hemolymph of *L. vannamei*, which is collected from the ventral sinus using a sterile syringe containing shrimp anticoagulant (27 mM Sodium citrate, 336 mM NaCl, 115 mM Glucose and 9 mM EDTA). The sample was centrifuged at 100×*g* for 10 min to remove the hemocytes. The resulting plasma was further centrifuged at 100,000×*g* (Optima L-80 XP Ultracentrifugation, BECKMAN, USA) for 4 h to remove the hemocyanin and dialyzed against 20 mM Tris-HCl (pH7.0) containing 0.15 M NaCl as described by Ariki et al. [28].

Hemocytes of normal shrimp were lysed for 10 min by IP Cell lysis buffer (Beyotime, Cnina) following the manufacturer's instruction. The samples from shrimp hemocytes, shrimp plasma, LVP and New Zealand white rabbit serum (negative control) were boiled in loading buffer and separated on 10% SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Millipore, Billerica, MA) by using standard methods. Membrane was blocked overnight at 4 °C in 5% skimmed milk in TBST (150 mM NaCl, pH 7.5, 20 mM Tris-HCl (pH8.2) and 0.05% Tween 20) and then incubated with 1/1000 diluted anti-Lv-C3L raised in rabbits (Beijing Protein Innovation Co., China) in TBST with 5% skimmed milk for 5 h at room temperature. After washing three times for 10 min with TBST and blocking 30 min using 2.5% skimmed milk, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (1/1000 diluted in TBST) was added. The membrane was incubated for 4 h, and unbound IgG was then washed four times for 15 min. The membrane was processed for detection of antibody binding using ECL Plus (Beyotime Institute of Biotechnology, China) and visualized with Eastman Kodak film according to the manufacturer's instructions.

2.9. Bacteriolytic and hemolytic activities assays

After overnight culturing and removing the supernatant medium by centrifuging, sediments of *V. parahaemolyticus* and *V. anguillarum* were activated by PBS and formed 2.0×10^3/ml PBS activated bacterial suspension of *V. parahaemolyticus* (PABSP) and *V. anguillarum* (PABSA), respectively. The samples used included either PBS, LVP, 0.068 μg/μl Lv-C3L antibody, heat inactivated LVP (56 °C for 30min) and LVP plus Lv-C3L antibody (34 μg Lv-C3L antibody/500 μL LVP). 0.5 ml of samples were mixed with 0.5 ml of PABSP and PABSA in different sterile tubes respectively and incubated at 28 °C for 6 h. After 0 h, 1 h, 3 h and 6 h incubated, 100 μL mixture in each group was cultured on TSA plate (PABSP group) and LB plate (PABSA group). The colonies were counted after 12 h of culture. A 2^{-1} fold dilution of PABSP and PABSA with PBS was used as a control. The assay was repeated in triplicate. Data are presented as arithmetic mean of parallel assays ± SE. Significance was set as $p < 0.05$.

Erythrocytes were separated from New Zealand white rabbit and made into a 2% suspension in PBS. The treatment groups contained the LVP, LPS (sigma, USA) plus LVP (1.0 μg LPS/ml LVP), Lv-C3L antibody plus LVP (4.9 μg Lv-C3L antibody/100 μL LVP), LPS plus Lv-C3L antibody and LVP (1.0 μg LPS/ml LVP, 4.9 μg Lv-C3L antibody/100 μL LVP), heat inactivated LVP (56 °C for 30min), heat inactivated LVP plus LPS (1.0 μg LPS/ml heat inactivated LVP) and1.0 μg/ml LPS. Solution from each group (0.5 ml) was mixed with 0.5 ml of 2% erythrocyte suspension in each sterile tube, respectively. After incubation for 5 h at 28 °C, the mixture was centrifuged at 10,000×*g* for 10 min. The absorbance of 100 μL supernatant was detected at 570 nm by using Infinite M200 PRO (Tecan, Switzerland). Serial dilutions of supernatant (2^0-2^{-5} fold) from each treatment group were analyzed in triplicate. PBS was used as the negative control and water was used as the positive control. The rate of hemolysis was calculated by: $(OD_{Sample}-OD_{PBS}) \times 100/(OD_{Water}-OD_{PBS})$.

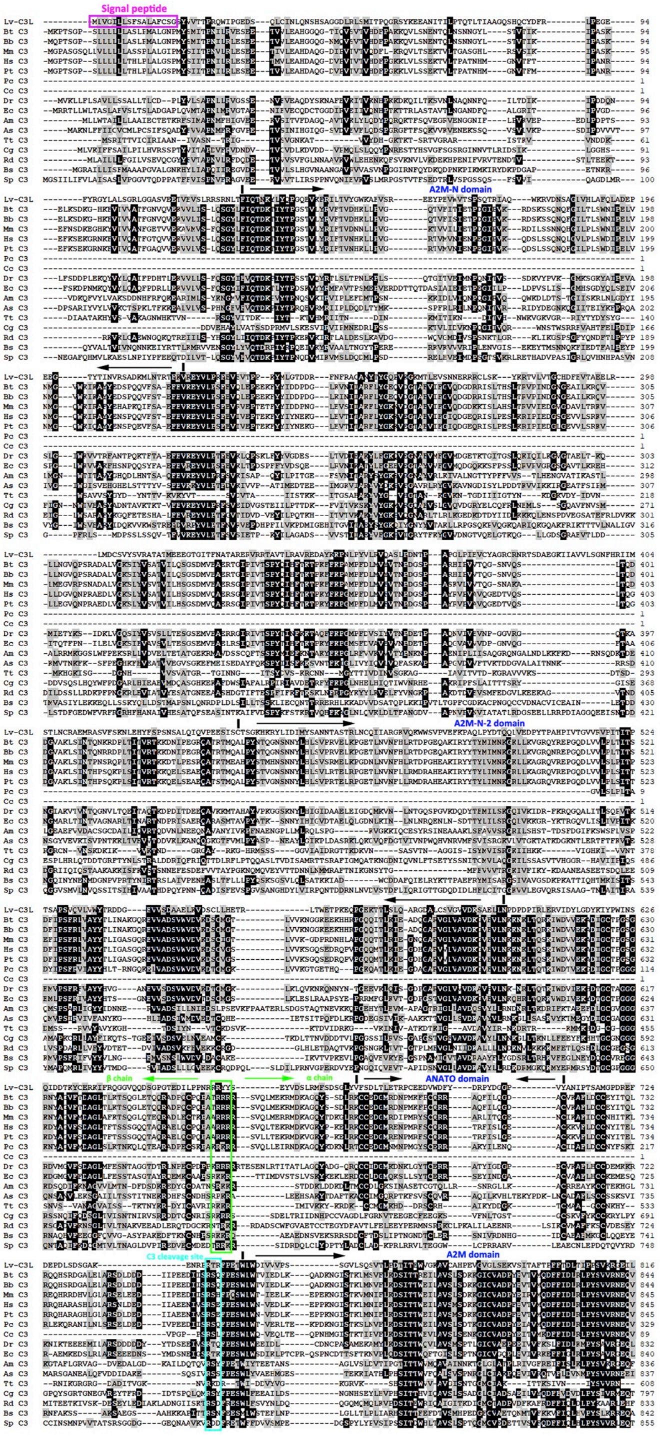

Fig. 2. Multiple alignment of Lv-C3L from *L. vannamei* with sixteen C3 family members from different species (obtained from the NCBI GenBank). They are Lv-C3L, Bt C3 (*Bos Taurus*, GenBank accession number: AKE14287.1), Bb C3 (*Bubalus bubalis*, AJW76887.1), Mm C3 (*Mus musculus*, AAH43338.1), Hs C3 (*Homo sapiens*, AAI50180.1), Pt C3 (*Pan troglodytes*, JAA28175.1), Pc C3 (*Phascogale calura*, AOF41418.1), Cc C3 (*Caiman crocodilus*, AHC72443.1), Dr C3 (*Danio rerio*, NP_571317.1), Ec C3 (*Epinephelus coioides*, ADU33222.1), Am C3 (*Acropora millepora*, ABK78771.2), As C3 (*Ammothea sp.*, BAR45600.1), Tt C3 (*Tachypleus tridentatus*, BAH02276.1), Cg C3 (*Crassostrea gigas*, NP_001292308.1), Rd C3 (*Ruditapes decussatus*, ACN37845.1), Bs C3 (*Botryllus schlosseri*, ALM04210.1) and Sp C3 (*Strongylocentrotus purpuratus*, NP_999686.1). Residues identical with the threshold of 50% in all sequences are shaded. The conserved and identical residues are represented by black shading, and conservative substitutions are represented by grey shading. The conserved five domains of C3 are shown above the alignment by bidirectional arrows. The signal peptide, predicted α-β junction, C3-convertase cleavage site, thiol-ester region and GLN-rich region were showed by line-box.

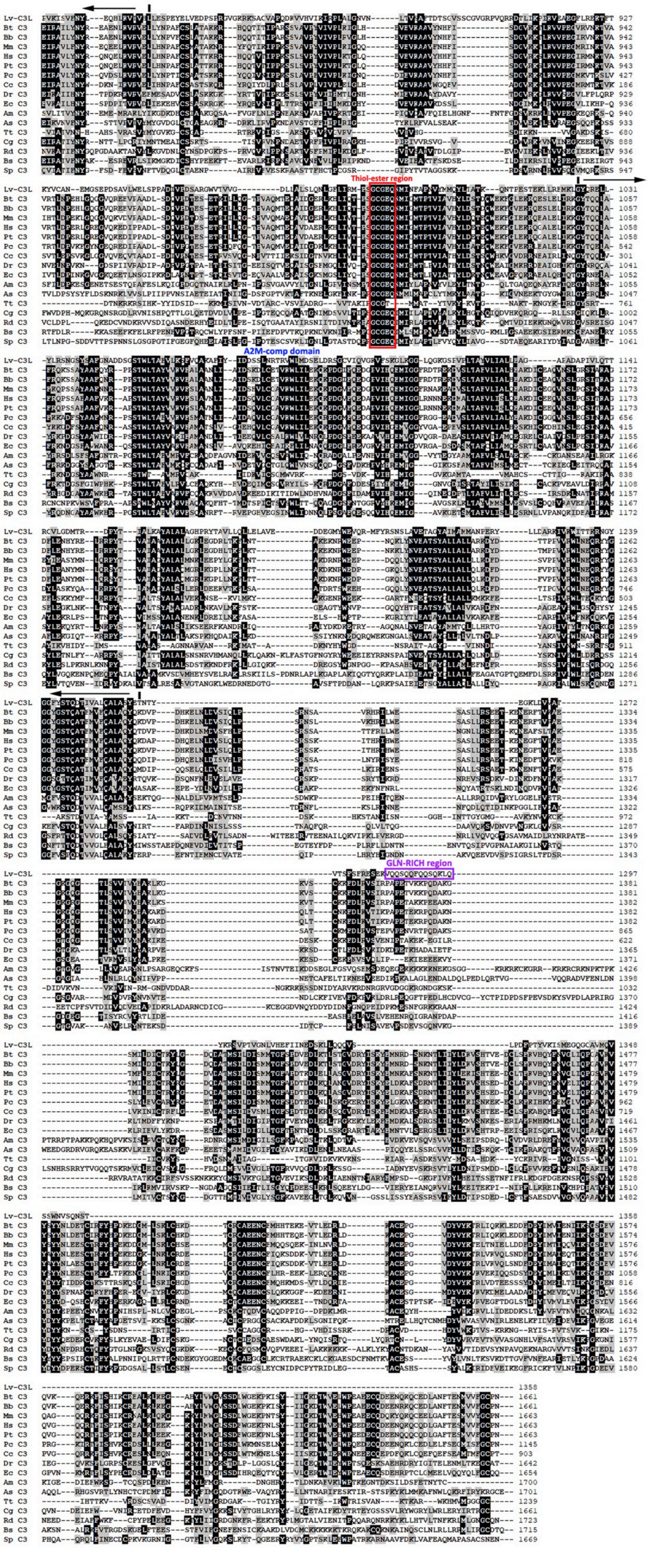

Fig. 2. (*continued*)

2.10. Preparation and optimization of double-strand RNA

To better understand the function of Lv-C3L, the interference effects of endogenous *Lv-C3L* on the propagation of WSSV in *L. vannamei* were detected. Primers with T7 promoter sequences, Lv-C3L-dsF and Lv-C3L-dsR (Table 1) were designed to amplify a 273-bp cDNA fragments of Lv-C3L as the template for dsRNA synthesis. Primers of EGFP-dsF and EGFP-dsR with T7 promoter sequences (Table 1) were used to amplify a 287-bp DNA fragment of EGFP gene as negative control. The PCR amplification program was as follows: denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s; final extension at 72 °C for 10 min. Linear double-stranded DNA (experimental and control group)with T7 promoter sequence were synthesized. The purified PCR products were used to synthesize the corresponding dsRNAs by using In Vitro Transcription T7 Kit (TaKaRa) for in vitro transcription reaction. DNA and redundant single-strand RNA was digested by DNaseI (5U/μL) and RNaseT1 (4U/μL) (TaKaRa). The dsRNAs were purified by Phenol/chloroform extraction and isopropyl alcohol precipitation. The concentration of synthesized dsRNAs were assessed by Nanodrop 2000. In order to optimize the silencing efficiency of *Lv-C3L* dsRNA, 42 healthy shrimps were divided into six groups in experimental group and control group, respectively. Shrimp in each group were injected with 15 μg *Lv-C3L* dsRNA or *EGFP* dsRNA for each individual. At 0 h, 12 h, 24 h, 36 h, 48 h and 72 h after interference, hemocytes of seven shrimps in each group were isolated for total RNA extraction. The transcription level of Lv-C3L was detected by qPCR with primers Lv-C3L qF and Lv-C3L qR as described in Section 2.6. Differential expression between different groups were assessed by statistical significance with a *t*-test ($p < 0.05$ and $p < 0.01$).

2.11. RNA interference and WSSV infection

2.11.1. WSSV copy number analysis after silencing of Lv-C3L

In order to explore the effect of *Lv-C3L* silencing on WSSV propagation, 40 shrimp were divided into 2 groups including dsEGFP group and ds*Lv-C3L* group. After optimizing of RNA interference effect, 15 μg dsEGFP and 15 μg ds*Lv-C3L* was injected into each shrimp in dsEGFP group and ds*Lv-C3L* group, respectively. At 12 h after dsRNA injection, 10^4 copies of WSSV dissolved in 10 μl TN buffer were injected into each individual. Hemocytes, gill and hepatopancreas of 15 individuals from each group were collected at 36 h after WSSV injection. The samples collected from each group were divided into three subgroups and frozen in liquid nitrogen for DNA and RNA extraction and WSSV copy number analysis. DNA were extracted from hemocytes and frozen tissues by using Rapid Animal Genomic DNA Isolation Kit (Sangon, China) following the manufacturer's instructions. The WSSV copy number in each DNA samples was quantified by qPCR with Gene-specific primers (W71F and W71R) and internal reference primers (TPI-qF and TPI-qR) (Table 1) according to the method described by Xue et al. [29]. The plasmid DNA containing a 121 bp fragment of VP28 gene from WSSV was constructed and transformed into *E. coli*. The plasmid was then extracted and quantified, and the copy number was calculated. Standard curve was constructed using 10-fold dilutions of the plasmid DNA ranging from 10^9 to 10^3. The qPCR was performed with the diluted plasmid DNA and the shrimp DNA under the following conditions: denaturation at 95 °C for 2 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The WSSV copy number per microlitre of shrimp DNA was calculated based on the standard curve.

2.11.2. Detection on the expression level of WSSV-VP28 gene after silencing of Lv-C3L

Total RNA was extracted from hemocytes, gill and hepatopancreas with Trizol reagent, assessed by electrophoresis in 1% agarose gel and quantified by NanoDrop 2000 spectrophotometer. The cDNA were synthesized by using GoScript ™ Reverse Transcription System (Promega). The expression of VP28 gene was analyzed by qPCR with primers VP28-qF and VP28-qR. The TPI gene, which served as a stably expressed reference, was quantified with primers TPI-qF and TPI-qR (Table 1). The qPCR for both genes was carried out as described in above.

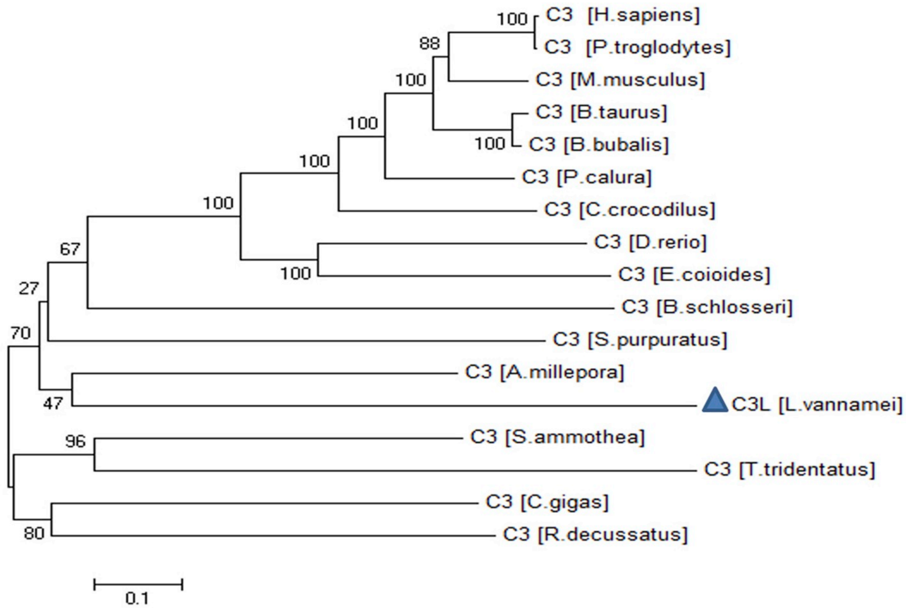

Fig. 3. Phylogenetic tree was constructed to analysis the homology of Lv-C3L with sixteen C3 family members (same as Fig. 2). A blue triangle was marked before Lv-C3L. The phylogenetic tree was constructed by ClustalW-generated multiple sequence alignment of amino acid sequences using neighbor-joining from the MEGA 5 package. The topological stability of the neighbor-joining trees was evaluated by 10,000 bootstrapping replications. The bar indicates the distance and the numbers at branches indicate the bootstrap values (%). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

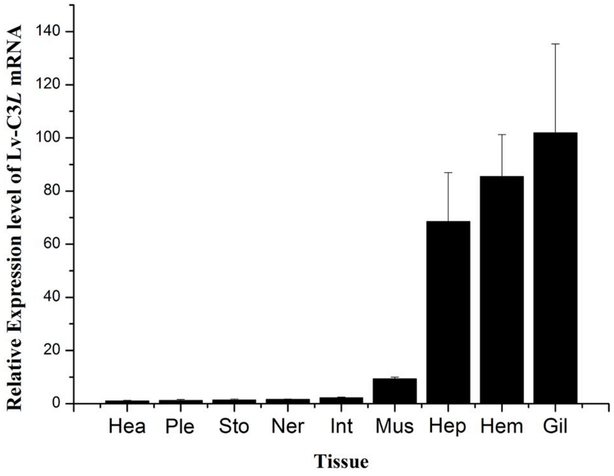

Fig. 4. Distribution of *Lv-C3L* mRNA by qRT-PCR in nine tissues from healthy shrimp *L. vannamei*. The results are representative of three independent experiments. Bars represented the mean ± SE (n = 3) for each tissue. Hea, heart; Ple, pleoplod; Sto, stomach; Ner, nerves; Int, intestines; Mus, muscles, Hep, hepatopancreas; Hem, hemocytes; Gil, gill.

2.11.3. Cumulative mortality after WSSV challenges in Lv-C3L-knockdown shrimp

Healthy shrimps were injected at the second abdominal segment with 15 μg dsRNA (*Lv-C3L* dsRNA in experimental group and *EGFP* dsRNA in control group). 12 h later, shrimps were injected again with 10 μl newly extracted WSSV particles (1 × 10^6 copies). Group without any injection was used as blank group. Three parallel subgroups (25 shrimps in each subgroup) were set in each group. Shrimps were cultured in recirculating aquaculture system with continuous aeration for about 7 days following injection. Cumulative mortality was recorded every day. Differences in mortality were tested using the Kaplan-Meier method (log-rank χ^2 test).

3. Results

3.1. The full-length cDNA of Lv-C3L and sequence analysis

The full length cDNA of Lv-C3L sequence was 4769 bp (GenBank accession number: MH638255) containing a 4077 bp open reading frame (ORF), which encodes 1358 amino acids. The full-length nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. The cDNA contained a 5′ untranslated region (UTR) of 69 bp, a 3′-UTR of 626 bp including a stop codon (TAA), putative polyadenylation consensus signal (AATAAA) and a poly (A) tail. The deduced protein has a calculated molecular mass of 150.3 KDa and a theoretical isoelectric point of 6.02. SignalP software analysis revealed that it contained a putative signal peptide of 17 amino acids. Eight potential N-glycosylation sites of Lv-C3L were identified (N^{49}, N^{107}, N^{234}, N^{306}, N^{364}, N^{865}, N^{1019} and N^{1059}).

3.2. Sequence comparison and phylogenetic analysis

SMART (Simple Modular Architecture Research Tool) and InterProScan analysis results show that Lv-C3L contains seven functional domains and motifs: A2M_N (α2-macroglobulin family N-terminal region, residues 108–202), A2M_N_2 (α2-macroglobulin family N-terminal region, residues 416–582), ANATO (anaphylatoxin-like domain, residues 664–693), A2M (α2-macroglobulin family domain, residues 729–819), Thiol-ester_C1 (thiol_ester bond-forming region, residues 958–987), A2M_comp (α-macroglobulin complement component, residues 1007–1243) and a GLN-RICH region (residues 1267–1280) (Fig. 2B). The highly conserved thioester (GCGEQ) region was located at amino acids 968–972 in Lv-C3L, which is a highly conserved region among invertebrate and vertebrate *C3* gene (Fig. 2B). Two peptide chains were predicted to be present in Lv-C3L protein, and were generated by motifs for an α-β junction (^{644}RRYY^{647}). C3a, as an important active fragment, was predicted in Lv-C3L by identification of the C3-convertase cleavage site R^{723}TR^{725}. Six highly conserved cysteine residues existed in C3a (Fig. 2A).

Seventeen different C3 sequences were aligned and the amino acid similarity was calculated. The evolutionary relationship between Lv-C3L and other C3 based on the phylogenetic tree was analyzed. The species used in the phylogenetic tree were Mammalia (*Homo sapiens*, GenBank: AAI50180.1; *Pan troglodytes*, JAA28175.1; *Mus musculus*, AAH43338.1; *Bos Taurus*, AKE14287.1; *Bubalus bubalis*, AJW76887.1; *Phascogale calura*, AOF41418.1), Amphibia (*Caiman crocodilus*, AHC72443.1), Fish (*Danio rerio*, NP_571317.1; *Epinephelus coioides*, ADU33222.1), Chordate (*Botryllus schlosseri*, ALM04210.1), Echinodermata (*Strongylocentrotus purpuratus*, NP_999686.1), Coelenterata (*Acropora millepora*, ABK78771.2), Arthropoda (*Ammothea sp.*, BAR45600.1), Arthropoda (*Tachypleus tridentatus*, BAH02276.1)

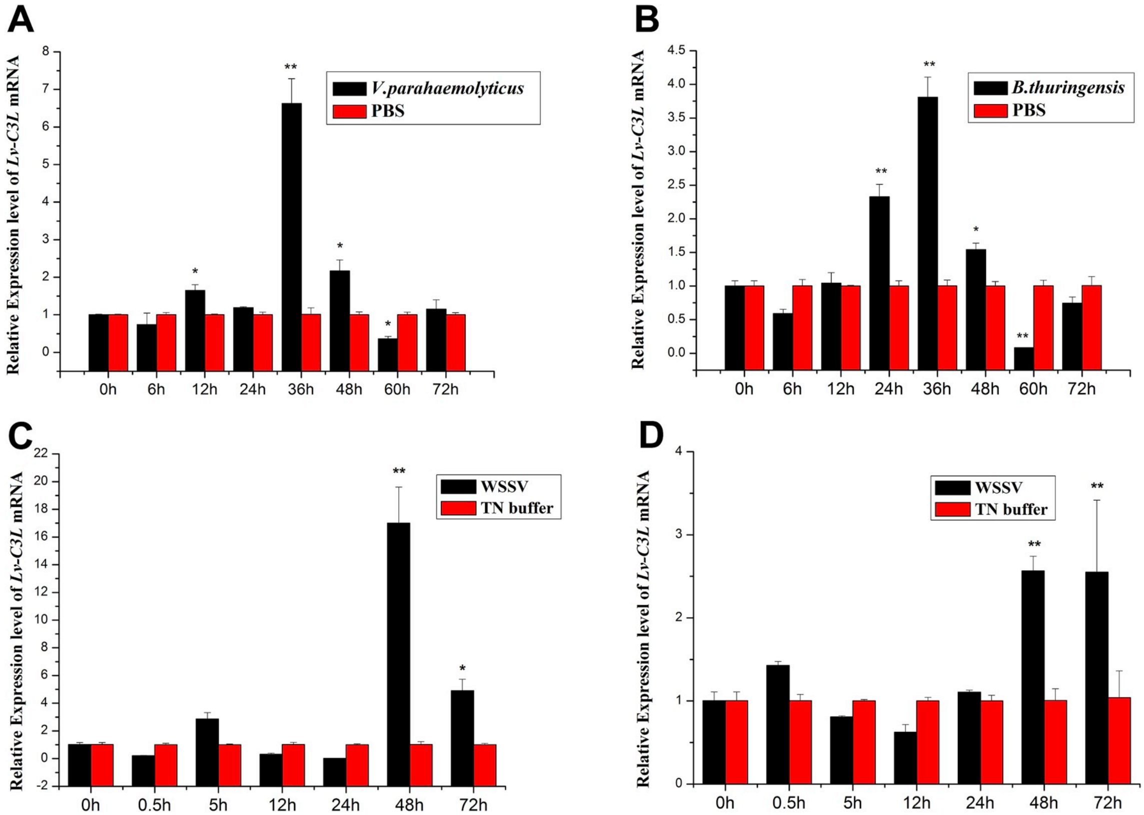

Fig. 5. Expression profile of *Lv-C3L* in shrimp hemocytes at different time post *V.parahaemolyticus* infection (A), *B.thuringensis* infection (B), in hemocytes (C) and gill (D) at different time post WSSV infection. *V.parahaemolyticus*, *V.parahaemolyticus* injection group; *B.thuringensis*, *B.thuringensis* injection group; PBS, phosphate-buffered saline injection group; WSSV, white spot syndrome virus injection group; TN buffer, TN buffer injection group. Stars (*) and double stars (**) indicate significant differences ($P < 0.05$) and extremely significant differences ($P < 0.01$) for the gene expression levels between experimental and control groups.

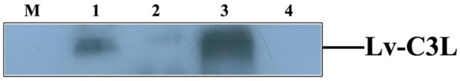

Fig. 6. Verification specific antibody of Lv-C3L. Lane M, protein marker; Lane 1, LVP; Lane 2, hemocytes of *L. vannamei*; Lane 3, plasma of *L. vannamei*; Lane 4, New Zealand white rabbit serum.

and Mollusca (*Crassostrea gigas*, NP_001292308.1, *Ruditapes decussatus*, ACN37845.1) (Fig. 3). C3s from different species were analyzed and formed two branches, grouped as vertebrates and invertebrates. Six C3 from mammalia and amphibia C3 are clustered. The C3s of fish, chordate and echinodermata are clustered together. Two C3s of mollusca are clustered. In invertebrate branch, Lv-C3L is most closely related to *Acropora millepora* C3, but not the Arthropoda *Tachypleus tridentatus* C3, although it lies close. *Tachypleus tridentatus* C3 and *Ammothea sp*. C3 are clustered and they also show certain similarities with Lv-C3L.

3.3. Distribution of Lv-C3L mRNA in shrimp tissues

The expression levels of *Lv-C3L* in different tissues were shown in Fig. 4 qRT-PCR analysis was performed on cDNA from nine tissues including heart, hepatopancreas, stomach, nerve, intestine, muscle, pleoplod, hemocytes and gill. The transcript of *Lv-C3L* was mainly detected in gill and hemocytes, low in the muscles. There was almost no expression in heart, hepatopancreas, stomach, nerve and intestine.

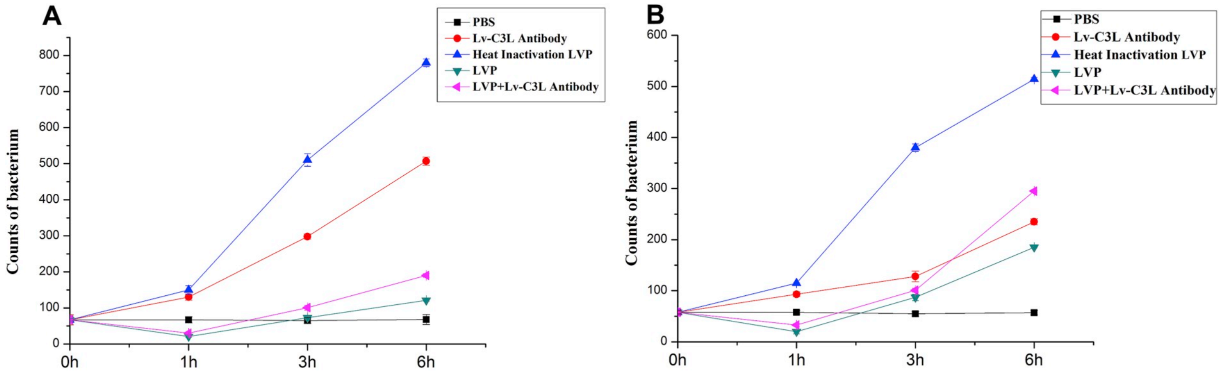

Fig. 7. Bacterial counts of *V.parahaemolyticus* (A) and *V. anguillarum* (B) at 0 h, 1 h, 3 h and 6 h after treatment with five groups: PBS, Lv-C3L antibody, heat inactivated LVP, LVP and LVP + Lv-C3L antibody. Boxes represent the mean ± SE ($n = 3$) for each sample.

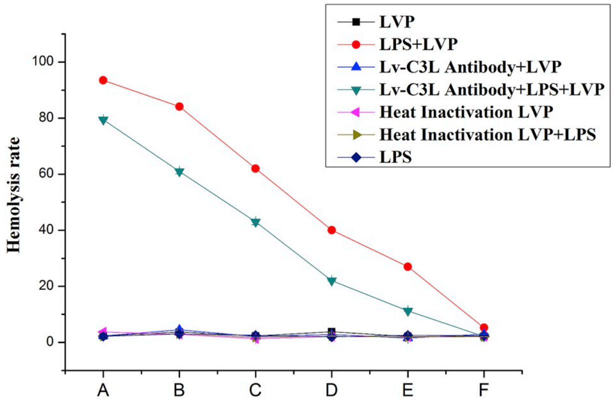

Fig. 8. The hemolytic rate of Lv-C3L on erythrocytes of New Zealand white rabbit in 5 h after treatment with each of seven groups: LVP, LPS + LVP, Lv-C3L antibody + LVP, Lv-C3L antibody + LPS + LVP, heat inactivated LVP, heat inactivated LVP + LPS and LPS. Letters A-F means 2^0-2^{-5} fold dilution of LVP, respectively. The A-F of LPS group represent the same conditions. Boxes represent the mean ± SE (n = 3) for each sample.

3.4. Expression profile of Lv-C3L gene in shrimp after pathogeny (bacterial and WSSV) challenge

The expression profiles of *Lv-C3L* in hemocytes and gill of shrimp after WSSV, *V.parahaemolyticus* and *B.thuringensis* challenge were detected. In hemocytes, expression levels of *Lv-C3L* were significantly upregulated at 36 and 48 hpi post *V.parahaemolyticus* infection (Fig. 5A) and upregulated at 24, 36 and 48 hpi post *B.thuringensis* infection (Fig. 5B). In bacterial challenge groups, expression levels of *Lv-C3L* were both downregulated at 60 hpi post infection. The expression levels of *Lv-C3L* were significantly upregulated at 48 and 72 h post WSSV infection (hpi) in hemocytes (Fig. 5C) and gill (Fig. 5D).

3.5. Bacteriolytic and hemolytic analysis in vitro

Western blot assay of Lv-C3L protein in shrimp hemocytes, shrimp plasma, LVP and New Zealand white rabbit serum showed that, at the protein level, Lv-C3L is detected in shrimp plasma and LVP, but not in the shrimp hemocytes and rabbit serum (Fig. 6). By Western blot analysis, the Lv-C3L antibody was used as a test verification that the Lv-C3L protein could trigger bacteriolytic and hemolytic activities. Bacteriolytic analysis showed that the group of LVP and LVP + Lv-C3L antibody were associated with bacteriolytic activation of *V.parahaemolyticus* (Fig. 7A) and *V.anguillarum* (Fig. 7B) significantly after 1 h incubation. The group of LVP is more antibiotic than group of LVP + Lv-C3L antibody.

Hemolytic analysis of Lv-C3L (Fig. 8) showed that hemolysis was occurred in two groups: LVP + LPS and LVP + Lv-C3L antibody + LPS. The hemolytic rate decreased with the fold dilution of LVP. Among all groups, the hemolytic rate of LVP + LPS was higher than that of LVP + Lv-C3 antibody + LPS. In addition, hemolysis was not observed in other groups including LVP, Lv-C3L antibody + LVP, heat inactivation LVP, heat Inactivation LVP + LPS and LPS.

3.6. Silencing of Lv-C3L affected in vivo virus propagation

RNA interference based on dsRNA was performed to study the function of Lv-C3L gene. The silencing efficiency of Lv-C3L was detected by qPCR at different time after injection with dsRNA, including 12, 24, 36, 48 and 72 h with dsRNA dosage 5 μg per shrimp. The mRNA level of Lv-C3L in hemocytess was remarkably downregulated at 36 h after dsRNA injection, while the EGFP dsRNA injection did not induce down-regulation of Lv-C3L. The optimal interference time is 36 h with 82.5% inhibition efficiency (Fig. 9A). It was used for further RNAi experiment. The WSSV copy number and relative expression level of WSSV-*VP28* gene were detected in shrimp after dsRNA and WSSV injection. At 24 hpi, the WSSV copy number in shrimp hemocytes, hepatopancreas and gill from dsEGFP group were about 2.2×10^4, 1.9×10^4 and 1.8×10^4 copies/μL shrimp DNA, while it was about 2.3×10^5, 9.2×10^4 and 9.4×10^4 copies/μL shrimp DNA from dsLv-C3L group, which was significantly higher ($P < 0.01$) than that of dsEGFP group (Fig. 9B). Among them, WSSV copy number in hemocytes was higher than that in hepatopancreas and gills. The relative expression level of WSSV-*VP28* gene showed the same features with that of WSSV copy number. In hemocytes, hepatopancreas and gill, relative expression level of *VP28* in the Lv-C3L knockdown group were much higher than that in control group ($P < 0.01$). The highest relative expression level of *VP28* was found also in hemocytes (Fig. 9C). As shown in Fig. 9D, the cumulative mortality of shrimps in Lv-C3L knockdown group increased rapidly from 2 days post WSSV challenge until 7 days. Cumulative mortality in the Lv-C3L dsRNA group by WSSV infection was significantly higher than that in control group (EGFP dsRNA) ($P < 0.05$). Final mortality rates were 90.3%, 71.7% and 0% for the Lv-C3L dsRNA, EGFP dsRNA and blank groups (without any injection), respectively. In addition, all shrimps in blank group survived.

4. Discussion

Complement pathway is an essential component of the innate immunity in many immunological processes in vertebrates and invertebrates. The origin of the complement system can be traced back from deuterostomes and protostome [4,13,30]. Research on the C3 gene had revealed that C3 plays an important role in innate immune system. In vertebrates, the molecular characterization and immune function of C3 have been reported [31,32]. However, there are much fewer reports of complement C3 in invertebrates. Molecular structure, functional research and regulation analysis on the complement components in shrimp are especially rare and need further exploration. Here, a new C3 like gene (Lv-C3L) was identified in *L. vannamei.* It participates in shrimp innate immunity and is responsive to pathogen infection. The bacteriolytic activity and hemolytic activity of Lv-C3L and its function during WSSV infection were also analyzed in this research.

The structure of complement C3 can offer insights into complement activation and regulation. Lv-C3L contained a putative signal peptide without transmembrane domain, indicating that it was synthesized and glycosylated in hemocytes and secreted to hemolymph plasma. The molecular structure of the C3 gene in invertebrates included eight conservative motifs suggested by Nonaka [33]. Lv-C3L contained six of these motifs, including the proteolytic cleavage site of ANATO. The C3 gene was considered to originate from cnidaria when the ANATO region was inserted in the C3 sequence [34]. However, for the lower sea urchin and coral, C3 molecules do not have ANATO domain. The A2M domain may play a pivotal role by endopeptidase inhibiting protease activity through encapsulation, covalent binding and adhesion reactions. Proteins containing A2M domain share several defining properties, which include the ability to inhibit proteases from all catalytic classes, the presence of a ‘bait region’ and a thiol ester, a similar protease inhibitory mechanism and the inactivation of the inhibitory capacity by reaction of the thiol ester with small primary amines [35]. The α-macroglobulin receptor (A2M_recep) domain and the Netrin C-terminal domain (C345C) did not exist in Lv-C3L. The thiol-ester region (GCGEQ) in invertebrates was described as an important motif for guiding C3 to fix on the surface of xenobiotics or pathogens [13]. In mammals, The A2M and C3/C4/C5 belong to the family of thiol containing protein. This highly consistent amino acid sequence of thiol-ester region was also found in Lv-C3L. The GLN-rich region might be involved in the interaction of this protein with other members of the heterogeneous nuclear ribonucleoprotein protein family [36] and transcriptional activation

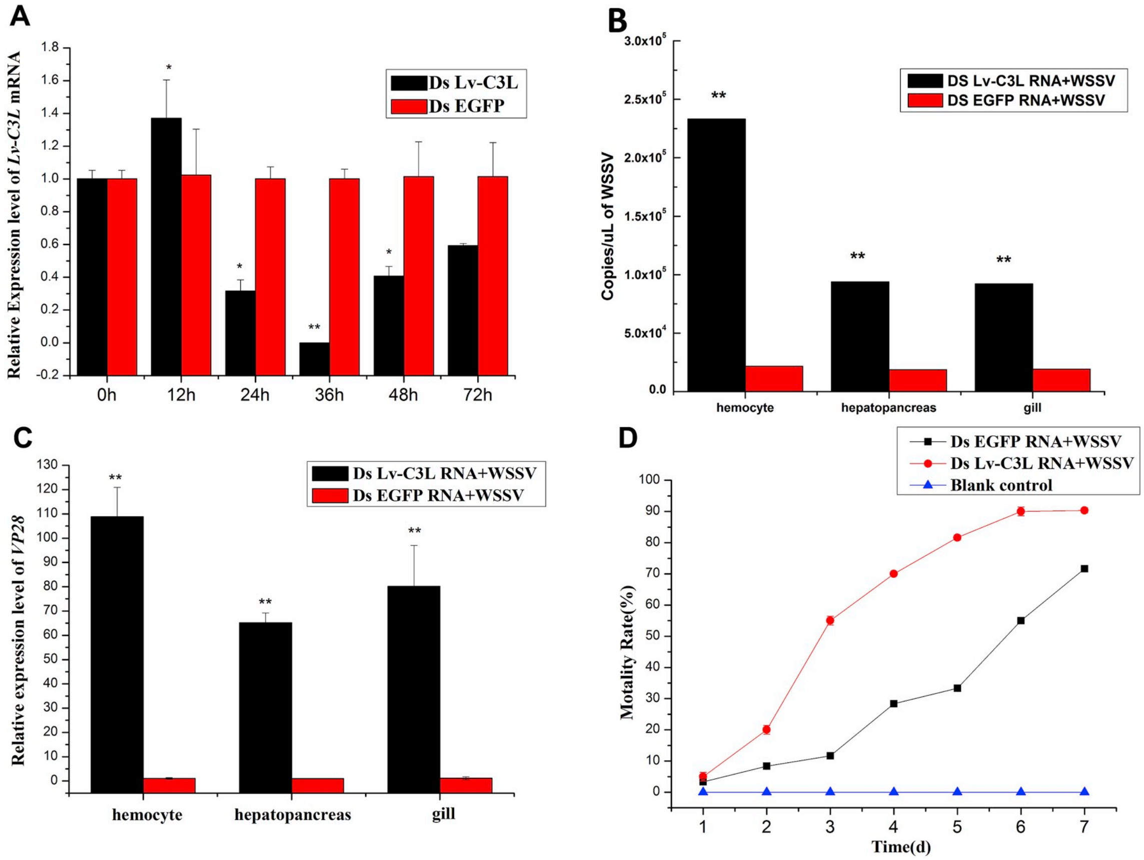

Fig. 9. RNA interference and function analysis of Lv-C3L during WSSV infection. (A) Expression level of Lv-C3L in shrimp hemocytes after injection dsRNAs in 0, 12, 24, 36, 48 and 72 h dsEGFP, injected with dsEGFP; dsLv-C3L, injected with dsRNA designed for Lv-C3L. (B) Amount of white spot syndrome virus (WSSV) particles in different shrimp tissues after silencing of Lv-C3L and WSSV infection. dsEGFP + WSSV, injected with dsEGFP and WSSV; dsLv-C3L + WSSV, injected with dsLv-C3L and WSSV. (C) Relative expression level of WSSV-VP28 gene in shrimp hemocytes, hepatopancreas and gill after silencing of Lv-C3L and WSSV infection. dsEGFP + WSSV, injected with dsEGFP + WSSV; dsLv-C3L + WSSV, injected with dsLv-C3L + WSSV. (D) Shrimp cumulative mortality following treatment with dsLv-C3L and infected by WSSV during 7 days. dsEGFP + WSSV, injected with dsEGFP + WSSV; dsLv-C3L + WSSV, injected with dsLv-C3L + WSSV; Blank control, without any injection. Stars (*) and double stars (**) show significant differences ($P < 0.05$) and extremely significant differences ($P < 0.01$) for the gene expression levels between groups.

[37]. In addition to typical conservative domains, Lv-C3L also contains this particular GLN-rich region.

Function could be predicted by analyzing characteristic regions. Though some C3 factor was consisted of three disulfide-linked polypeptide chains [28,38], most typical C3 is a β2 glycoprotein containing two subunit peptide chains structure (α and β) and belongs to the thioester-containing protein (TEP) superfamily [10]. The potential sites of α-β peptide chains junction was located in ^{644}RRYY^{647} of Lv-C3L by sequence comparison. The γ peptide chains were not predicted in Lv-C3L sequence. It indicated that Lv-C3L show similar characteristics compare with normal C3 reported in other species. It was speculated that two peptides (scissions in α-β junction) exist in the mature Lv-C3L protein. The activation of the lectin pathway can lead to the activation of C3 molecule. C3a is the most important active fragment of the complement system. The C3-convertase cleavage site has been found as a conserved region (LXR) in most vertebrates. However, this site is not conserved in invertebrates, as reported for *H. roretzi* TSR [39], *A. japonicas* RXR [40] and *S. constricta* VNR [18]. In the corresponding position, a "RTR" site was found in Lv-C3L. It might be involved in the formation of C-terminal residues of Lv-C3La. The alpha2-macroglobulin (A2M) and the complement components C3 and C4 are related proteins derived from a common ancestor. From selacean, bony fish to higher animal, *C3* gene occurred in the common ancestor of A2M. C3 were conserved throughout evolution in different species from cnidarian to human. Phylogenic analysis results showed that Lv-C3L was clustered with coelenterata *A. millepora* C3, which showed that they might have a close evolutionary relationship. Compare with vertebrate C3, arthropoda *T. tridentatus* C3 and *Ammothea sp.*C3 also showed close evolutionary relationship with Lv-C3L.

In order to know whether Lv-C3L was involved in immune response in shrimp, its spatial distribution and temporal expression during pathogen infection were detected. C3 protein had been produced from liver cells or macrophages in mouse or humans [41] and from hemocytes in *Botryllus schlosseri* [42]. In this research, *Lv-C3L* mainly expressed in hemocytes and gill constitutively. In crustaceans, hemocytes play extremely important roles in defense not only by direct sequestration and killing of infectious agents, but also by synthesis, storage and exocytosis of a battery of bioactive molecules [43]. They could phagocytose foreign material and exert important functions in the innate immunity system. Expression of Lv-C3L protein from LVP in hemolymph revealed a similar pattern. As one of important molecules in the hemolymph, C3 might plays a crucial role in crustacean innate immunity. Gill is the first barrier to shrimp immune defense. High expression of *Lv-C3L* in gills of *L. vannamei* might be due to their immune defense function and infiltrating hemocytes in branchial filaments [21–23]. This result indicates that it can be mainly expressed in tissues or cells which might be involved in defense response.

The complement system functions during infection and directly mediates pathogen elimination. As a major component of complement pathway in humans, C3 links innate immunity to adaptive immune system and also plays an essential role in several diseases [44]. Though the innate immunological function of C3 had been preliminarily reported by bacterial challenge, the function of C3 has not been described in detail in invertebrate. In our study, *Lv-C3L* showed different responses to bacteria and WSSV. In sea cucumber, AjC3-2 and AjC3 genes expression increased significantly at 6 h after the LPS challenge [40]. The mRNA expression levels of three C3 isoforms in dojo loach were up-regulated in the gill, skin, liver and spleen after infection with *Aeromonas hydrophila* [45]. *A. hydrophila* challenge could also resulted in significant up-regulated expression of C3 transcripts in both liver and kidney post-infection in *Labeo rohita* [46]. The genus *Vibrio* is considered to be one of most devastating pathogens among shrimp bacteria disease. *V. parahaemolyticus* and *B.thuringensis* were main pathogens of hepatopancreatic necrosis (HPNS) in *L. vannamei*. At 36 h and 48 h post *V.parahaemolyticus* infection and 24 h, 36 h, 48 h post *B.thuringensis* infection, the expression level of *Lv-C3L* gene in hemocytes was significantly upregulated. The data showed that *Lv-C3L* might be a mid-late response gene to bacterial infection in shrimp. Moreover, there was a significant decrease of *Lv-C3L* expression at 60 h post bacterial infection. The possible reason for the decrease might be that proliferation of pathogenic bacteria inhibit C3 gene expression and the serpins may have controlled degradation and activity of the complement proteins [47]. In *Aedes aegypti*, complement-related proteins could control the flavivirus infection by inducing antimicrobial peptides [48]. Complement factor transcripts were immunologically upregulated by lipopolysaccharide (LPS), *Streptococcus iniae*, rock bream iridovirus (RBIV) and poly I:C in spleen tissue in rock bream [49]. In human astrocytes, immunodeficiency virus type 1 could induces expression of complement factors [50]. However, Hepatitis B virus inhibits the expression of complement C3 and C4 in vitro and *in vivo* [51]. WSSV is one of the most serious viral pathogen of shrimp farming industry resulted in huge economic losses in the worldwide. In our study, at 48 h and 72 h post WSSV infection, the expression level of *Lv-C3L* gene in hemocytes and gill were significantly upregulated. The data indicated that *Lv-C3L* might be a late phase response gene to WSSV infection. These results indicated that *Lv-C3L* gene play a pivotal role in immune responses to the WSSV and G^+/G^- bacterial infection. The different response pattern of *Lv-C3L* following bacterial and WSSV challenges maybe an indication that multiple response pathways exist in shrimps. Besides *V. parahaemolyticus*, *B.thuringensis* and WSSV, the expression of *Lv-C3L* responsive to other stimulants is worthy to further study.

Bacteriolytic and hemolytic activities of C3 protein had been analyzed in human saliva [52] and amphioxus humoral fluid [53]. The bacteriolytic activity analysis of Lv-C3L showed that LVP group and LVP plus Lv-C3L antibody group had significant bacteriolysis, and the bacteriostatic effect of LVP group was more obvious than that of the LVP plus Lv-C3L antibody group. Moreover, both of V.*parahaemolyticus* and *V.anguillarum* analysis groups still expressed bacteriolytic activities when Lv-C3L proteins were inhibited by its antibody. These results indicated the effective immune function of Lv-C3L in bacteriolysis. There may be other bacteriolytic factors in LVP, such as antimicrobial peptides, lysozyme and isoforms of Lv-C3L. Counts of bacterium in all groups increased after 1 h (Fig. 7A and B). It was considered that Lv-C3L and other proteins might consumed by bacterium and lose bacteriolytic ability. Hemolysis is a crucial experiment to identify the function of C3 in invertebrates. Comparing with results of bacteriolytic analysis, similar result was obtained in hemolytic activity analysis of Lv-C3L. Furthermore, Lv-C3L protein is a heat-labile protein just like C3 from other species [12,33] and needs to be activated by antigen like LPS or bacterium [28,54]. After Lv-C3L restrained by its antibody in “Lv-C3L antibody + LPS + LVP” group, the hemolysis was still observed in experiments though the hemolysis rate was lower than that of the “LPS + LVP” group. It indicated that some other factors with hemolytic activity which had similar function to Lv-C3L exist in LVP and were activated by LPS. The gap between two groups may indicate the hemolytic activity of Lv-C3L. The above results revealed that Lv-C3L plays a vital role in bacteriolysis and hemolysis during innate immune response.

Combining WSSV copy number analysis with WSSV-*VP28* gene expression profile analysis, interference of *Lv-C3L* gene by double-strand RNA could obviously promote the *in vivo* propagation of WSSV in shrimp. It can be inferred that Lv-C3L could inhibit the replication of WSSV in shrimp. Similarly, cumulative mortality in the Lv-C3L dsRNA group by WSSV infection was significantly higher than that in control group (EGFP dsRNA). Though previous studies showed that complement-related proteins could control the virus infection [48], this is the first report in crustaceans that Lv-C3L is involved in antiviral immune response. These results collectively suggested that Lv-C3L may play an important protective role against WSSV and activation of complement pathway including C3 could restrict the propagation of WSSV in shrimp. In conclusion, a novel complement C3 like gene (Lv-C3L) with full-length cDNA sequence was identified in shrimp *L. vannamei* in the present study. Its phylogenetic pattern, expression profile in healthy tissues and in pathogen challenged, bacteriolytic and hemolytic activities were also explored. *Lv-C3L* was responsive to *V. parahaemolyticus*, *B.thuringensis* and WSSV infection and could regulate the *in vivo* propagation of WSSV. This is the first report on key complement components in shrimp. It is speculated that complicated complement response cascade may exist in shrimp. These results collectively indicated that the complement pathway in shrimp might participate in bacterial and WSSV infection. Further research on the identification of molecular mechanism concerning C3 deposition on bacteria and WSSV, membrane rupture and the special complement pathway are in progress. These studies would help increase understanding the function of C3 gene in invertebrate immune system and give new evidence for further research on the primary complement molecules in invertebrates.

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References

[1] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, R.A. Ezekowitz, Phylogenetic perspectives in innate immunity, Science 284 (5418) (1999) 1313–1318.
[2] R. Medzhitov, C. Janeway Jr., Innate immune recognition: mechanisms and pathways, Immunol. Rev. 173 (2000) 89–97.
[3] K. Murphy, C. Weaver, Janeway's Immunobiology (9TH Edition), Garland Science, Taylor & Francis Group, LLC, 2016.
[4] T. Fujita, Evolution of the lectin-complement pathway and its role in innate immunity, Nat. Rev. Immunol. 2 (5) (2002) 346–353.
[5] M.J. Walport, Complement. First of two parts, N. Engl. J. Med. 344 (14) (2001) 1058–1066.
[6] M.J. Walport, Complement. Second of two parts, N. Engl. J. Med. 344 (15) (2001) 1140–1144.
[7] M.C. Carroll, The complement system in regulation of adaptive immunity, Nat. Immunol. 5 (10) (2004) 981–986.
[8] M. Chen, M.R. Daha, C.G. Kallenberg, The complement system in systemic autoimmune disease, J. Autoimmun. 34 (3) (2010) 276–286.
[9] A.W. Dodds, S.K. Law, The phylogeny and evolution of the thioester bond-containing proteins C3, C4 and alpha 2-macroglobulin, Immunol. Rev. 166 (1998) 15–26.
[10] A.A. Abdul, K. Gunasekaran, J.E. Volanakis, S.V. Narayana, G.J. Kotwal, H.M. Murthy, The structure of complement C3b provides insights into complement activation and regulation, Nature 444 (7116) (2006) 221–225.
[11] S.E. Degn, S. Thiel, Humoral pattern recognition and the complement system, Scand. J. Immunol. 78 (2) (2013) 181–193.
[12] Z. Gao, M. Li, J. Wu, S. Zhang, Interplay between invertebrate C3a with vertebrate macrophages: functional characterization of immune activities of amphioxus C3a, Fish Shellfish Immunol. 35 (4) (2013) 1249–1259.

[13] Y. Zhu, S. Thangamani, B. Ho, J.L. Ding, The ancient origin of the complement system, EMBO J. 24 (2) (2005) 382–394.

[14] D. Ricklin, G. Hajishengallis, K. Yang, J.D. Lambris, Complement: a key system for immune surveillance and homeostasis, Nat. Immunol. 11 (9) (2010) 785–797.

[15] K. Tagawa, T. Yoshihara, T. Shibata, K. Kitazaki, Y. Endo, T. Fujita, T. Koshiba, S. Kawabata, Microbe-specific C3b deposition in the horseshoe crab complement system in a C2/Factor B-dependent or -independent manner, PLoS One 7 (5) (2012) e36783.

[16] O. Simakov, F. Marletaz, S.J. Cho, E. Edsinger-Gonzales, P. Havlak, U. Hellsten, et al., Insights into bilaterian evolution from three spiralian genomes, Nature 493 (7433) (2013) 526–531.

[17] G. Zhang, X. Fang, X. Guo, L. Li, R. Luo, F. Xu, et al., The oyster genome reveals stress adaptation and complexity of shell formation, Nature 490 (7418) (2012) 49–54.

[18] M. Peng, D. Niu, F. Wang, Z. Chen, J. Li, Complement C3 gene: expression characterization and innate immune response in razor clam *Sinonovacula constricta*, Fish Shellfish Immunol. 55 (2016) 223–232.

[19] F. Li, J. Xiang, Recent advances in researches on the innate immunity of shrimp in China, Dev. Comp. Immunol. 39 (1–2) (2013) 11–26.

[20] F. Li, J. Xiang, Signaling pathways regulating innate immune responses in shrimp, Fish Shellfish Immunol. 34 (4) (2013) 973–980.

[21] Y.C. Liu, F. H Li, B. Dong, B. Wang, W. Luan, X.J. Zhang, et al., Molecular cloning, characterization and expression analysis of a putative C-type lectin (*Fclectin*) gene in Chinese shrimp *Fenneropenaeus chinensis*, Mol. Immunol. 44 (4) (2007) 598–607.

[22] Y.C. Liu, F.H. Li, B. Wang, B. Dong, X.J. Zhang, J.H. Xiang, A serpin from Chinese shrimp *Fenneropenaeus chinensis* is responsive to bacteria and WSSV challenge, Fish Shellfish Immunol. 26 (3) (2009) 345–351.

[23] Y.C. Liu, F.H. Li, B. Wang, B. Dong, Q.L. Zhang, W. Luan, et al., A transglutaminase from Chinese shrimp (*Fenneropenaeus chinensis*), full-length cDNA cloning, tissue localization and expression profile after challenge, Fish Shellfish Immunol. 22 (5) (2007) 576–588.

[24] J. Rodriguez, V. Boulo, E. Mialhe, E. Bachere, Characterization of shrimp hemocytes and plasma components by monoclonal-antibodies, J. Cell Sci. 108 (Pt 3) (1995) 1043–1050.

[25] 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 (1–2) (2005) 63–67.

[26] S. Xue, Y. Liu, Y. Zhang, Y. Sun, X. Geng, J. Sun, Sequencing and *de novo* analysis of the hemocytes transcriptome in *Litopenaeus vannamei* response to white spot syndrome virus infection, PLoS One 8 (10) (2013) e76718.

[27] Y. Zhang, Y. Sun, Y. Liu, X. Geng, X. Wang, Y. Wang, et al., Molt-inhibiting hormone from Chinese mitten crab (*Eriocheir sinensis*): cloning, tissue expression and effects of recombinant peptide on ecdysteroid secretion of YOs, Gen. Comp. Endocrinol. 173 (3) (2011) 467–474.

[28] S. Ariki, S. Takahara, T. Shibata, T. Fukuoka, A. Ozaki, Y. Endo, et al., Factor C acts as a lipopolysaccharide-responsive C3 convertase in horseshoe crab complement activation, J. Immunol. 181 (11) (2008) 7994–8001.

[29] S. Xue, W. Yang, J. Sun, Role of chymotrypsin-like serine proteinase in white spot syndrome virus infection in *Fenneropenaeus chinensis*, Fish Shellfish Immunol. 34 (2) (2013) 403–409.

[30] M. Nonaka, F. Yoshizaki, Primitive complement system of invertebrates, Immunol. Rev. 198 (2004) 203–215.

[31] D. Grossberger, A. Marcuz, L. Du Pasquier, J.D. Lambris, Conservation of structural and functional domains in complement component C3 of Xenopus and mammals, Proc. Natl. Acad. Sci. U.S.A. 86 (4) (1989) 1323–1327.

[32] N.J. Lynch, S.U. Khan, C.M. Stover, S.M. Sandrini, D. Marston, J.S. Presanis, et al., Composition of the lectin pathway of complement in *Gallus gallus*: absence of mannan-binding lectin-associated serine protease-1 in birds, J. Immunol. 174 (8) (2005) 4998–5006.

[33] M. Nonaka, The complement C3 protein family in invertebrates, Invertebr. Surviv. J. 8 (1) (2011) 21–32.

[34] L. Cerenius, S. Kawabata, B.L. Lee, M. Nonaka, K. Söderhäll, Proteolytic cascades and their involvement in invertebrate immunity, Trends Biochem. Sci. 35 (10) (2010) 575–583.

[35] L. Sottrup-Jensen, Alpha-macroglobulins: structure, shape, and mechanism of proteinase complex formation, J. Biol. Chem. 264 (20) (1989) 11539–11542.

[36] M. Budini, E. Buratti, C. Stuani, C. Guarnaccia, V. Romano, L. De Conti, et al., Cellular model of TAR DNA-binding protein 43 (TDP-43) aggregation based on its C-terminal Gln/Asn-rich region, J. Biol. Chem. 287 (10) (2012) 7512–7525.

[37] D. Albani, L.S. Robert, Cloning and characterization of a *Brassica napus* gene encoding a homologue of the B subunit of a heteromeric CCAAT-binding factor, Gene 167 (1–2) (1995) 209–213.

[38] T. Fujii, T. Nakamura, S. Tomonaga, Component C3 of hagfish complement has a unique structure: identification of native C3 and its degradation products, Mol. Immunol. 32 (9) (1995) 633–642.

[39] M. Nonaka, K. Azumi, X. Ji, C. Namikawa-Yamada, M. Sasaki, H. Saiga, et al., Opsonic complement component C3 in the solitary ascidian, *Halocynthia roretzi*, J. Immunol. 162 (1) (1999) 387–391.

[40] Z. Zhou, D. Sun, A. Yang, Y. Dong, Z. Chen, X. Wang, et al., Molecular characterization and expression analysis of a complement component 3 in the sea cucumber (*Apostichopus japonicus*), Fish Shellfish Immunol. 31 (4) (2011) 540–547.

[41] M.K. Liszewski, J.P. Atkinson, Complement regulators in human disease: lessons from modern genetics, J. Intern. Med. 277 (3) (2015) 294–305.

[42] F. Nicola, B. Loriano, Morula cells as key hemocytes of the lectin pathway of complement activation in the colonial tunicate *Botryllus schlosseri*, Fish Shellfish Immunol. 63 (2017) 157–164.

[43] V.J. Smith, J.R.S. Chisholm, Non-cellular immunity in crustaceans, Fish Shellfish Immunol. 2 (1) (1992) 1–31.

[44] J.R. Delanghe, R. Speeckaert, M.M. Speeckaert, Complement C3 and its polymorphism: biological and clinical consequences, Pathology 46 (1) (2014) 1–10.

[45] Y. Xu, Y. Yu, X. Zhang, Z. Huang, H. Li, S. Dong, et al., Molecular characterization and expression analysis of complement component 3 in dojo loach (*Misgurnus anguillicaudatus*), Fish Shellfish Immunol. 72 (2018) 484–493.

[46] K. Pushpa, P. Gireesh-Babu, K.V. Rajendran, C.S. Purushothaman, S. Dasgupta, M. Makesh, Molecular cloning, sequencing and tissue-level expression of complement C3 of *Labeo rohita* (Hamilton, 1822), Fish Shellfish Immunol. 40 (1) (2014) 319–330.

[47] S.H. Rooijakkers, J.A. van Strijp, Bacterial complement evasion, Mol. Immunol. 44 (1–3) (2007) 23–32.

[48] X. Xiao, Y. Liu, X. Zhang, J. Wang, Z. Li, X. Pang, et al., Complement-related proteins control the flavivirus infection of *Aedes aegypti* by inducing antimicrobial peptides, PLoS Pathog. 10 (4) (2014) e1004027.

[49] G.I. Godahewa, N.C. Perera, S.D. Bathige, B.H. Nam, J.K. Noh, J. Lee, Complement factor D homolog involved in the alternative complement pathway of rock bream (*Oplegnathus fasciatus*): molecular and functional characterization and immune responsive mRNA expression analysis, Fish Shellfish Immunol. 55 (2016) 423–433.

[50] C. Speth, G. Stöckl, I. Mohsenipour, R. Würzner, H. Stoiber, C. Lass-Flörl, et al., Human immunodeficiency virus type 1 induces expression of complement factors in human astrocytes, J. Virol. 75 (6) (2001) 2604–2615.

[51] C. Zhu, H. Song, F. Xu, W. Yi, F. Liu, X. Liu, Hepatitis B virus inhibits the expression of complement C3 and C4, *in vitro* and *in vivo*, Oncol. Lett. 15 (5) (2018) 7459–7463.

[52] A. Andoh, Y. Fujiyama, T. Kimura, H. Uchihara, H. Sakumoto, H. Okabe, et al., Molecular characterization of complement components (C3, C4, and factor B) in human saliva, J. Clin. Immunol. 17 (5) (1997) 404–407.

[53] Z. Li, S. Zhang, C. Wang, Q. Pang, Complement-mediated killing of *Vibrio* species by the humoral fluids of amphioxus *Branchiostoma belcheri*: implications for a dual role of O-antigens in the resistance to bactericidal activity, Fish Shellfish Immunol. 24 (2) (2008) 215–222.

[54] J.C. Tam, S.R. Bidgood, W.A. McEwan, L.C. James, Intracellular sensing of complement C3 activates cell autonomous immunity, Science 345 (6201) (2014) 1256070.