Fish and Shellfish Immunology 84 (2019) 252–258

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

An MBT domain containing anti-lipopolysaccharide factor (PtALF8) from *Portunus trituberculatus* is involved in immune response to bacterial challenge

Xiaodong Wu[a,b], Yanwei Huang[a], Zuoben Yu[a], Changkao Mu[a,*], Weiwei Song[a], Ronghua Li[a], Lei Liu[a], Yangfang Ye[a], Ce Shi[a], Chunlin Wang[a,**]

[a] Key Laboratory of Applied Marine Biotechnology, Ministry of Education, Ningbo University, Ningbo, 315211, China
[b] Collaborative Innovation Center for Zhejiang Marine High-efficiency and Healthy Aquaculture, Ningbo University, Ningbo, 315211, China

ARTICLE INFO

Keywords:
Anti-lipopolysaccharide factors
Recombinant protein
Antibacterial activity
Quantitative PCR

ABSTRACT

Anti-lipopolysaccharide factors are effective antimicrobial peptides that can bind and neutralize lipopolysaccharide (LPS). In the present study, a new sequence encoding for ALF (designated as PtALF8) was cloned by suppression subtractive hybridization method using ovary of swimming crab *Portunus trituberculatus* as material. The full-length cDNA of PtALF8 consisted of 531 bp with an ORF of 348 bp encoding a peptide of 115 amino acids containing a putative signal peptide of 19 amino acids. The mature PtALF8 had a predicted molecular weight (MW) of 11.28 kDa and theoretical isoelectricpoint (pI) of 5.11. The PtALF8 contains an MBT domain which was not found in the other 7 isoforms of ALF reported in *P. trituberculatus*. Unlike most ALFs expressed in hemocytes, PtALF8 transcript was predominantly detected in hepatopancreas. After challenge with *Vibrio alginolyticus*, the temporal expression level of PtALF8 transcript in hemocytes reached the highest level at 3 h, then decreased to the lowest level at 24 h, and started to increase at 48 h. The recombinant protein showed antimicrobial and bactericidal activity against several bacteria, such as Gram-positive bacteria, *Staphylococcus aureus*, *Micrococcus luteus* and Gram-negative bacteria, *V. alginolyticus*, indicated that the PtALF8 isoform might play protective function against invading bacteria in *P. trituberculatus*.

1. Introduction

It had been demonstrated that antimicrobial host defense was dependent both on innate and adaptive components [1]. The innate immune system, provides the first line of constitutively pre-existing host defense against infection [2], is a universal and ancient form of host defense [3]. Antimicrobial peptides, which served as a fundamental role in the successful evolution of complex multicellular organisms, were reported to widespreadly distribute throughout the animal and plant kingdoms [4,5].

Anti-lipopolysaccharide factors (ALFs) are one type of AMPs that can bind and neutralize lipopolysaccharides (LPS), forming a key effector molecule of the innate immune system in crustaceans [6]. The first ALF was isolated from the hemocytes of *Tachypleus tridentatus* and *Limulus polyphemus*, which was reported to be able to inhibit the lipopolysaccharide (LPS)-mediated activation of the *Limulus* coagulation system [7]. In crustaceans, several ALF molecules were studied [8–10], the molecular characterization and antibacterial spectrum were demonstrated.

The swimming crab, *Portunus trituberculatus*, mainly distribute in the coastal waters of Japan, Korea and China [11], is a commercially important fishery and aquaculture species in China [12]. Outbreaks of infectious diseases especially that caused by bacteria limited the development of this industry. It is important to understand the characterization of immunity and to establish strategies for prophylaxis and control of diseases. As an invertebrate, crabs possess a rapid and efficient innate system to recognize and destroy non-self material [13]. In the past decade, several immune related genes about *P. trituberculatus* were reported [14–16] including several isoforms of ALF [17–20].

In the present study, a new isoform of ALF was cloned from suppression subtractive hybridization library of *P. trituberculatus*, which share higher similarity with ALF from the *Scylla paramamosain* than that of the other reported isoforms of *P. trituberculatus*. To know more about the characterization of this new isoform of ALF, the mature peptides

* Corresponding author. Ningbo University, 818 Fenghua Rd., Ningbo 315211, PR China.
** Corresponding author. Ningbo University, 818 Fenghua Rd., Ningbo 315211, PR China.
E-mail addresses: muchangkao@nbu.edu.cn (C. Mu), wangchunlin@nbu.edu.cn (C. Wang).

https://doi.org/10.1016/j.fsi.2018.10.016
Received 11 May 2018; Received in revised form 1 October 2018; Accepted 5 October 2018
Available online 06 October 2018

Table 1
Primers used in the experiment.

primer	Sequence (5′-3′)	Sequence information
P1	TTGACTTCGGAGCCATAGAGAT	PtALF8 specific primer (for full-length cDNA cloning)
P2	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT	Universal primer (for full-length cDNA cloning)
P3	GTCACGCATCTCGCTGCTTCT	Real-time PCR primer
P4	CCTTGTTCACCACCCTAGTCCT	Real-time PCR primer
P5	TCACACACTGTCCCCATCTACG	Beta-actin
P6	ACCACGCTCGGTCAGGATTTTC	Beta-actin
P7	CATATGCAAGACTACTTGAAAGAAGAACT	Recombinant primer
P8	CTCGAGTTAGTGGTGGTGGTGGTGCTCT AGCAAGGGTTGGGCGT	Recombinant primer

Fig. 1. Nucleotide and deduced amino acid sequences of PtALF8. The signal peptide is shaded with gray. The MBT domain is underlined. The two Cysteine (C^{48} and C^{69}) that involved in disulfide formation were enclosed and bolded.

were recombinant and the antibacterial activity of the recombinant protein, tissue distribution of transcription and temporal changes following *V. alginolyticus* challenge were studied.

2. Materials and methods

2.1. Animals and SSH cDNA library

The tested crabs were cultured in Xinyi corporation of Ningbo, China. The ovary from mature and immature female crabs were used for constructing suppression subtractive hybridization (SSH) cDNA libraries [21]. From the SSH cDNA library, an EST shared high similarity with ALF from *Scylla paramamosain* (with identity of 78.2%), then this EST was used for primer design to clone the full-length cDNA sequence of PtALF8.

2.2. Cloning of the full-length cDNA of PtALF8

One specific primer P1 (Table 1) was designed based on the sequence of EST to clone the full-length cDNA of PtALF8 from *P. trituberculatus*. PCR reaction to get the 3′ end of PtALF8 was performed using sense primer P1 and reverse primer P2 (Table 1). The specific PCR products were cut and gel-purified before being cloned into pMD19-T vector (Takara, Japan) and sequenced on an ABI3730 Automated Sequencer (Applied Biosystem).

2.3. Sequence and phylogenetic analysis

The homology of nucleotide and protein sequences was searched by using BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.gov/blast). SMART (http://smart.embl-heidelberg.de/) was used to analyze the deduced amino acid sequence. The presence and location of signal peptide was predicted with SignalP 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignment of the ALFs was performed with the Multiple Alignment show program (http://www.bio-soft.net/sms/). Phylogenic NJ tree was constructed with Mega 6 software package [22].

2.4. Quantitative analysis of PtALF8 transcript expression

The mRNA expression of PtALF8 in hepatopancreas, gill, muscle and hemocytes of unchallenged crabs, and the temporal changes of PtALF8 in hemocytes of crabs with *V. alginolyticus* challenge were determined by quantitative real-time RT-PCR. Total RNA was extracted from tissues of swimming crab using Trizol (Invitrogen) and the first strand of cDNA was synthesized using oligo (dT)-adaptor as primer. The cDNA used for quantitative real-time RT-PCR was diluted 10 times with DEPC-treated water. The real-time RT-PCR was carried out in a total volume of 20 μL, containing 10 μL of 2 × GoTaq® qPCR Master Mix (Applied Promega), 2 μL of the diluted cDNA, 0.4 μL of each primer (10 mmol L^{-1}), 7.2 μL of DEPC-water. Two gene-specific primers P3 and P4 (Table 1) were designed for the quantitative analysis. The β-actin from *P. trituberculatus* amplified with primers P5 and P6 (Table 1), was used as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The PtALF8 relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method [23]. Multiple-group comparisons were evaluated using analysis of one-way (ANOVA) and Duncan's test in the SPSS software (SPSS, Chicago, IL, USA). Differences were defined as significant at $P < 0.05$.

2.5. Expression and purification of recombinant PtALF8

PCR fragment encoding the mature peptide of PtALF8 was amplified using gene-specific primers P7 and P8 (Table 1). For the convenience of cloning, an *Nde* I site was added to the 5′ end of P7 and an *Xho* I site was added to the 5′ end of P8 before the stop codon. PCR products were digested completely by restriction enzymes *Nde* I and *Xho* I (NEB, USA), and then subcloned into the *Nde* I/*Xho* I sites of expression vector pET-21a (+) (Novagen, Germany). The recombinant plasmid (pET-21a-PtALF8) was transformed into *E. coli Origami* (DE3) (Novagen) and subjected to DNA sequencing. The parent vector without insert fragment was selected as negative control. After sequencing to ensure in-frame insertion, positive transformants of PtALF8 and negative control were incubated in LB medium (containing 100 mg/ml ampicillin) at 37 °C with shaking at 220 rpm. When the culture reached OD_{600} of 0.4–0.6, isopropyl-b-D-thiogalactosidase (IPTG) was added to the final concentration of 1 mM, and incubated for additional 5 h under the same conditions. Cells were harvested by centrifugation at 8500 *g* for 5 min at 4 °C, and suspended in 50 mM Tris containing 5 mM EDTA, 50 mM NaCl, and 5% Glycerol (pH7.9). After being sonicated at 4 °C for 60 min, the PtALF8 and negative control sample were purified by HisTrap Chelating Columns (Amersham Biosciences, Sweden) according to the manufacturer's instruction.

The purified proteins were collected and analyzed for their purity on

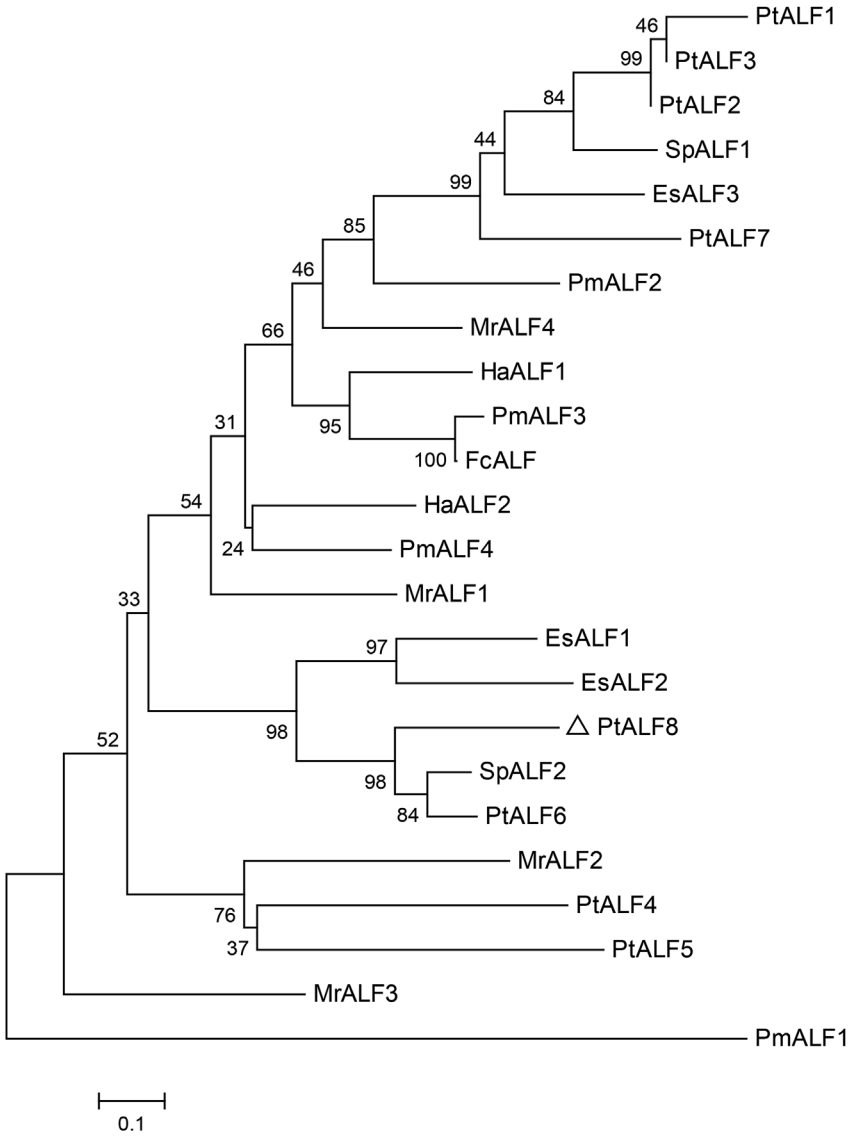

Fig. 2. Phylogenetic tree based on sequence of ALF from different decapod. This tree is constructed by the neighbor-joining (NJ) algorithm using the Mega 6 program based on multiple sequence alignment by ClustalW. Bootstrap trials were replicated 1000 times to derive the confidence value. The protein sequences used for phylogenetic analysis are as follows: FcALF (AAX63831) from *Fenneropenaeus chinensis*; ALF1 (ABP96982) and SpALF2 (ADT71676) from *Scylla paramamosain*; EsALF1 (ABG82027), EsALF2 (ACY25186) and EsALF 3 (ADZ46233) from *Eriocheir sinensis*; HaALF1 (ACC94268) and ALF2 (ACC94269) from *Homarus americanus*; PmALF1 (AEO16982), PmALF2 (ABP73291), PmALF3 (ABP73289) and PmALF4 (ADM21460) from *Penaeus monodon*; MrALF1 (AFW04304), MrALF2 (AFW04305), MrALF3 (AFW04306) and MrALF4 (ACN50186) from *Macrobrachium rosenbergii*; PtALF1 (HM627757), PtALF2 (HM627758), PtALF3 (GQ165621), PtALF4 (JF756050), PtALF5 (JF756051), PtALF6 (JF756052) and PtALF7 (AFA42345) from *Portunus trituberculatus*.

15% SDS-PAGE. The concentration of the recombinant protein was determined using a BCA Protein Assay Kit (Beyotime) according to the manufacturer's protocol.

2.6. Antibacterial activity of the recombinant PtALF8

Antibacterial activity were carried out against two Gram-positive bacteria (*Staphyloccocus aureus* and *Micrococcus luteus*), three Gram-negative bacteria (*E. coli* (JM109), *Aeromonas hydrophila* and *Vibrio alginolyticus*) and a fungus (*Pichia pastoris* GS115). The minimal inhibitory concentration (MIC) was determined as methods described by Hancock (http://cmdr.ubc.ca/bobh/methods/). Serial two-fold dilutions of synthesis polypeptide were made with corresponding growth medium in sterilized 96-well microtiter plates, repeat the procedure down to column 10 only. The test strains were cultured at corresponding temperatures in corresponding growth medium until the cfu was 0.5–1 × 10^8. Five microliter of bacteria and a fungus of 0.5 × 10^5 cfu/mL were inoculated to each well except blank column. Then the mixtures were cultured at 28 °C and 37 °C individually for 24 h at 600 nm for Gram-positive bacteria or 560 nm for Gram-negative bacteria and fungus of each well was read with ELISA reader. The assay was done with triplicates in three independent experiments and the MIC value was recorded as Casteels et al. [24]. After the MIC determination, aliquots of 100 μL from each well that does not show any bacterial growth after incubation were streaked onto nutrient agar followed by incubation at 28 °C and 37 °C individually for 24 h. The lowest concentration that kills 100% of the initial bacterial population showing no colonies on the nutrient agar was recorded as the MBC.

3. Results

3.1. Molecular characterization of PtALF8

A 531 bp nucleotide sequence representing the complete cDNA sequence of PtALF8 was acquired. The sequence was deposited in GenBank under accession number KJ081863. The complete sequence of PtALF8 cDNA contained a 5′ untranslated region (UTR) of 37 bp, a 3′ UTR of 146 bp with a polyA tail, and an open reading frame (ORF) of 348 bp encoding a polypeptide of 115 amino acids. Signal P analysis revealed that a signal peptide of 19 amino acids was identified with a predicted cleavage site between Ser^{19} and Gln^{20}, resulting an 11.28 kDa mature protein with theoretical isoelectric point of 5.11. The deduced amino acid sequence of PtALF8 was shown in Fig. 1. SMART program analysis revealed that PtALF8 contained a typical MBT domain (from Gln^{20} to Ser^{83}).

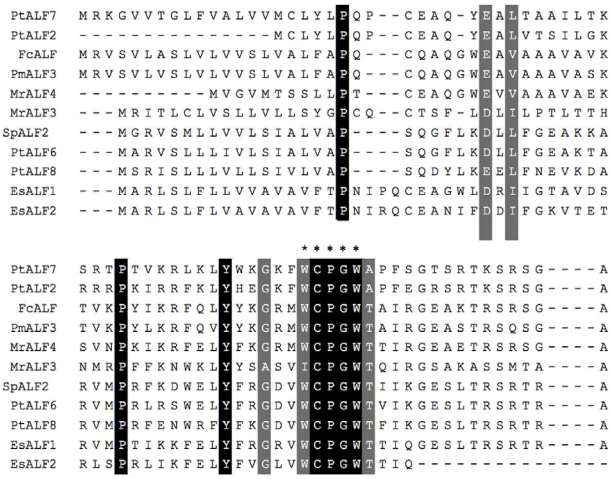

Fig. 3. Multiple sequence alignment of PtALF8 and other ALF. The conserved residues were shaded with black, and the consensus patterns were marked with stars. The sequences are as follows: SpALF2 (*Scylla paramamosain*, ADT71676), EsALF1 (ABG82027) and EsALF2 (ACY25186) from *Eriocheir sinensis*, MrALF4 (ACN50186) and MrALF3 (AFW04306) from *Macrobrachium rosenbergii*, FcALF (*Fenneropenaeus chinensis*, AAX63831), PtALF2 (HM627758), PtALF6 (JF756052) and PtALF7 (AFA42345) from *Portunus trituberculatus*, PmALF3 (*Penaeus monodon*, ABP73289).

3.2. Multiple sequence alignment and phylogenetic analysis

The deduced amino acid sequence of PtALF8 shared high similarity with anti-lipopolysaccharide factor from other species, such as 77.4% identity with SpALF1 from *Scylla paramamosain* (ADT71676) and 76.5% identity with PtALF6 from *P. trituberculatus* (JF756052). A phylogenic tree was constructed based on PtALF8 and anti-lipopolysaccharide factor from other decapods (Fig. 2). In the NJ tree, the PtALF8 was firstly clustered with that of SpALF1 from *Scylla paramamosain* (ADT71676) and PtALF6 from *P. trituberculatus* (JF756052) before being clustered with other anti-lipopolysaccharide factor. Protein sequences aligned using the ClustalW program embedded in the program Mega6 indicated that a consensus pattern of W(T)CPG(S)WT(A) existed in all the reported ALF (Fig. 3).

3.3. The three dimensional structure of PtALF8

Based on the high similarity with other ALFs, the potential tertiary structures of PtALF8 was established using the SWISS-MODEL prediction algorithm based on the template 2jobA. The deduced 3D structure of PtALF8 consists of three α-helices packed against a four-stranded β-sheet, in which, β2 and β3 are connected at the bottom by a disulfide bridge (Cys^{48}-Cys^{69}). The predicted 3D structure is similar to the NMR structure of ALF3 from *Penaeus monodon*, they are both shown in Fig. 4.

3.4. Expression, purification and identification of PtALF8

The recombinant plasmid pET-21a-PtALF8 was transformed and expressed in *E. coli* Origami (DE3) as described above. After IPTG induction, SDS-PAGE was used to analysis the whole cell lysate, and a distinct band with a molecular weight about 12 kDa (Fig. 5) was observed, which was consistent with the predicted molecular mass of fusion protein.

3.5. Antibacterial activity of the recombinant PtALF8

The recombinant PtALF8 was evaluated for its antibacterial activity against microorganisms by the MIC and MBC assay described in Materials and Methods. The purified rPtALF8 could inhibit the growth

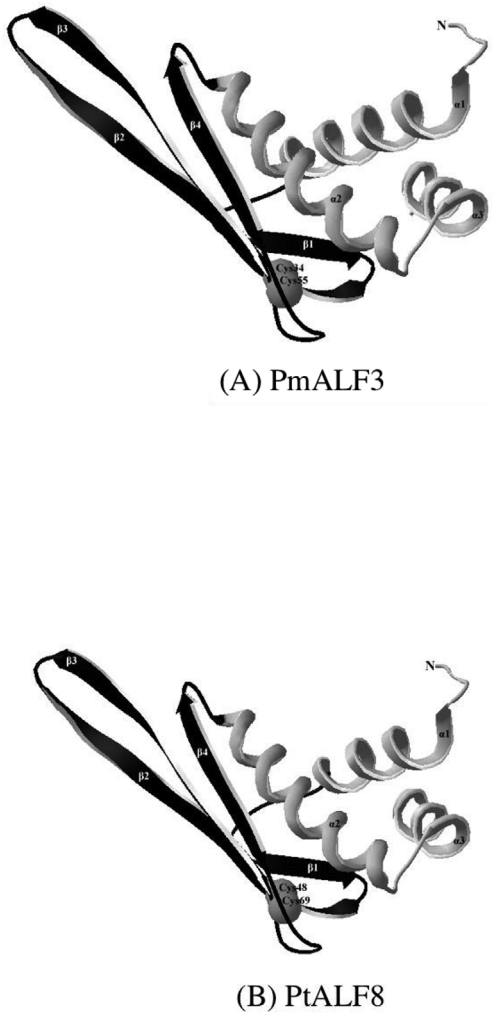

Fig. 4. Potential tertiary structure of PtALF8 and PmALF3 established using the SWISS-MODEL prediction algorithm. The α-helic were gray and the β-sheets were black. (A) PmALF3, (B) PtALF8.

of Gram-positive bacteria, such as *Staphylococcus aureus*, *Micrococcus luteus* and Gram-negative bacteria, *V. alginolyticus*, *Aeromonas hydrophil* and *Escherichia coli* with minimal inhibitory concentration of 0.69, 0.69, 0.34, 1.38 and 0.69 μM, respectively (Table 2). However, antibacterial activity was not detected against the *Pichia pastoris* GS115 (Table 2). In addition, the MBC values revealed that synthetic PtALF8 showed bactericidal effects against Gram-positive bacteria and Gram-negative bacteria (Table 2).

3.6. Tissue distribution of the PtALF8 transcription

mRNA expression of PtALF8 in the tissues of healthy crabs, including hemocytes, gill, hepatopancreas, eyestalk, and muscle, the quantitative real-time PCR was employed. PtALF8 was mainly expressed in the hepatopancreas, and with a lower level of expression in the hemocytes, but PtALF8 was almost undetected in gills, eyestalk and muscle (Fig. 6).

3.7. Expression pattern of PtALF8 in hemocytes after V. alginolyticus challenge

The temporal mRNA expression of PtALF8 transcripts in hemocytes post *V. alginolyticus* challenge was shown in Fig. 7. PtALF8 was significantly up-regulated after the *V. alginolyticus* challenge, reached the highest level at 3 h, and then declined at 6 h. As time progressed, the

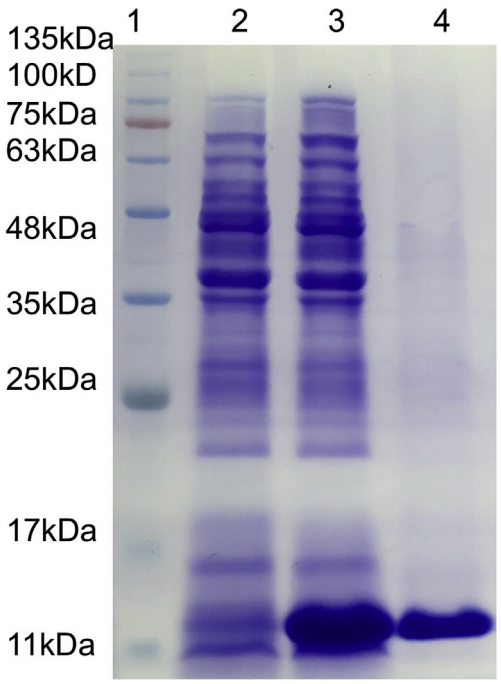

Fig. 5. SDS-PAGE analysis of PtALF8. Lane 1: protein molecular standard; lane 2: uninduced control without IPTG induction; lane 3: expression of PtALF8 after IPTG induction; lane 4: purified PtALF8.

Table 2
Antimicrobial activity of rPtALF8 derived from *Portunus trituberculatus*. MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; ND: not detectable.

Microorganisms	MIC(μM)	MBC(μM)
Gram-positive bacteria		
Micrococcus luteus	0.69	1.38
Staphylococcus aureus	0.69	1.38
Gram-negative bacteria		
Vibrio alginolyticus	0.34	0.69
Aeromonas hydrophila	1.38	2.76
Escherichia coli	0.69	1.38
Fungus		
Pichia pastoris GS115	ND	ND

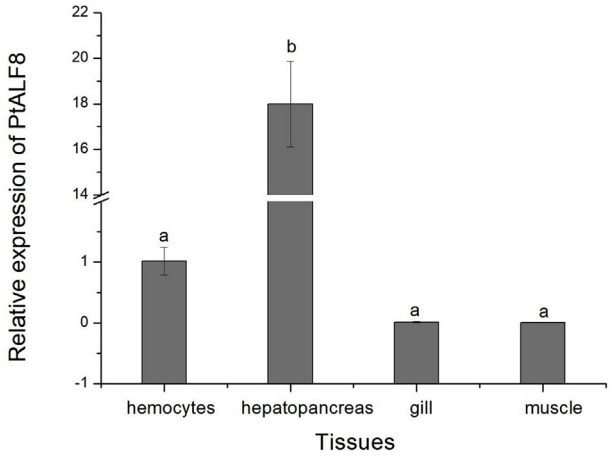

Fig. 6. Tissue distribution of the PtALF8 transcription detected by qRT-PCR. Each bar represents the mean ± S.E. (n = 3). Different letters represent significant differences ($P < 0.05$).

expression level was decreased gradually and reached the lowest point at 24 h post-injection. After that, it increased gradually at 48 h post-injection.

4. Discussion

Molecules that bind LPS and neutralize its biological effects or enhance its clearance could have important clinical applications [25]. In *P. trituberculatus*, 7 isoforms of anti-lipopolysaccharide factor (ALF) have been described [18]. In the present study, a new ALF isoform containing an MBT domain is cloned from *P. trituberculatus*. The PtALF8 shares high sequence similarity with other anti-lipopolysaccharide factor, such as 77.4% identity with SpALF1 and 76.5% identity with PtALF6. The predicted 3D structure is similar to the NMR structure of ALF3 from *Penaeus monodon* [26] and the crystal structure of ALF from *Limulus polyphemus* [25]. In addition, it contains the consensus pattern of 'WCPGWT', the highly two conserve cysteine residues and the putative LPS-binding domain, which were found in other reported ALF. All these suggest that PtALF8 can be classified as a member of ALF family. MBT domain was firstly reported in *lethal* (3) *maLignant brain tumor* [1 (3)*mbt*] gene from *Drosophila* [27]. It can recognize and bind histone marks and are in general found in proteins that bind to chromatin to repress transcription of endogenous and reporter genes, after being recruited to their promoters in vivo [28]. The MBT domain was not found in other ALF genes, even other reported ALF from *P. trituberculatus* [18] and the ALF2 from *S. paramamosain* [29]. So, the PtALF8 was suggested to be a new ALF isoform.

ALFs are small basic single polypeptides of about 100 amino acids with two conserved cysteine residues forming a disulfide bond that constrains a β-hairpin [30]. The LPS-binding domain, are formed between two conserved cysteine residues, which form a disulphide loop, and contain a cluster of positively charged residues [26]. These typical structures are reported in other seven PtALF isoforms, which is closely associated with the biological activities of ALFs. Distinct from them, PtALF8 consists of three α-helices packed against a four-stranded β-sheet, that may lead to different functions.

Several studies on crustaceans have shown that recombinant ALFs of different species are efficient against various bacteria [31]. Antimicrobial activity of PtALF8 was analyzed to better understand the molecular function involved in the immune system. The result indicated that PtALF8 could inhibit the growth of Gram-positive bacteria and Gram-negative bacteria at lower concentration but bactericidal at higher concentration, and the growth inhibitory effects of the peptide are due to bactericidal activity. The recombinant PtALF1, PtALF4 and PtALF5 protein have inhibition activity only against Gram-negative bacteria. Compared to the reported PtALFs, PtALF8 exhibits a stronger antibacterial activity against toward Gram-negative and Gram-positive bacteria [18–20]. However, contrast to ALFs from *Procambarus clarkii* [32] and *E. sinensis* [33], antifungal activity against *P. pastoris* in rPtALF8 was not detectable. While PtALF8 display a negative net charge at a physiological pH and are rather anionic in contrast with most of the ALF isoforms identified so far. Therefore, the stronger antibacterial activity may due to the MBT domain involved in transcriptional repression [28] contained in PtALF8, which is not found in other reported PtALFs. The mechanism of MBT domain in antibacterial function is not clear, the recombinant PtALF8 may interfere with bacterial transcription by interfering with the transcription of bacterial cell membrane proteins, resulting in a reduction in the amount of protein expressed, and then increased cell membrane permeability and impeding bacterial growth.

Most ALF genes including PtALF1-3 and PtALF5 are mainly expressed in hemocytes [10,17,20,29], PtALF4 was mainly expressed in the eyestalk and PtALF6 was dominantly expressed in gills. In the present study, hepatopancreas is the dominant expression tissue of PtALF8, which is consistent with that observed in MrALF5 and MrALF7 from giant freshwater prawn, *Macrobrachium rosenbergii* [34]. All these

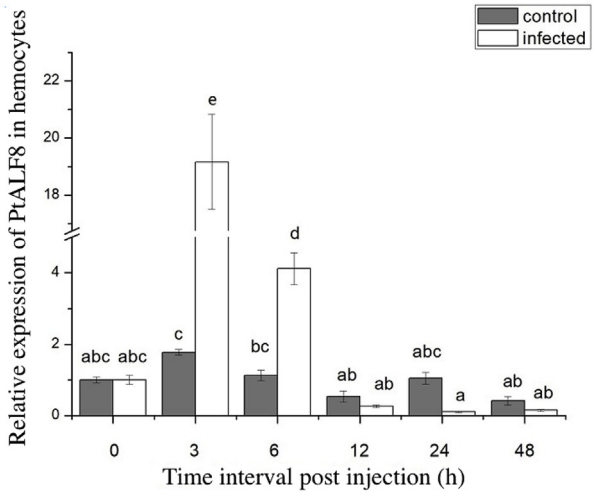

Fig. 7. Temporal expressions of PtALF8 transcript in hepatopancreas after *V. alginolyticus* challenge. The mRNA expression of PtALF8 (relative to β-actin) in the hemocytes in response to *V. alginolyticus* (white bars) or saline control (black bars) at 0, 3, 6, 12, 24 and 48 h post injection was determined by qRT-PCR. Data are represented as means ± S.E. (n = 3). Different letters represent significant differences ($P < 0.05$).

data suggest that ALF proteins could be expressed in a wide range of tissues and might provide multiple immune functions [19]. The higher PtALF8 transcripts in hepatopancreas and hemocytes supported its possible immune defense functions, since hemocytes and hepatopancreas in crustacean are considered to play important roles in immune defense not only by direct sequestration and killing of foreign invaders, but also by synthesis and exocytosis of bioactive molecules [35].

Different from the expression pattern of the other PtALF isoforms, the expression of PtALF8 transcript is up-regulated and reaches significant peak after *V. alginolyticus* challenge at 3 h. Then, the expression decreased to the lowest level at 24 h and started to increase at 48 h. It suggested that different ALF isoforms might have different operating times against pathogens. In the present study, the PtALF8 mRNA level in the pathogens challenge group was significantly higher ($P < 0.05$) than that of blank at 3 h post stimulation, which suggested that PtALF8 might be an acute-phase protein induced by pathogens challenge and probably played important roles in fighting bacterial and fungal infection.

5. Conclusion

In conclusion, a new isoform of ALF (PtALF8) from *P. trituberculatus* was cloned and characterized. The PtALF8 showed inhibitory and bactericidal activity on growth of Gram-positive bacteria and some Gram-negative bacteria. The future studies will attempt on developing its protection role in the *P. trituberculatus* against diseases from bacterial challenge. In addition, we will explore potential possibility in bacterial disease control and health management of crab aquaculture.

Acknowledgments

We thank members of our lab for their input and stimulating discussion and helpful comments on this manuscript. This work was supported by the National Natural Science Foundation of China (41676140), Major Agriculture Program of Ningbo (2017C110007), Modern technology system of agricultural industry (CARS-46), the Scientific Research Foundation of Graduate School of Ningbo University, and the K C Wong Magana Fund in Ningbo University.

References

[1] J.A. Hoffmann, F.C. Kafatos, C.A. Janeway, et al., Phylogenetic perspectives in innate immunity, Science 284 (1999) 1313–1318.
[2] D. Yang, A. Biragyn, D.M. Hoover, et al., Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense, Annu. Rev. Immunol. 22 (2004) 181–215.
[3] C.A. Janeway Jr., R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216.
[4] J.A. Tincu, S.W. Taylor, Antimicrobial peptides from marine invertebrates, Antimicrob. Agents Chemother. 48 (2004) 3645–3654.
[5] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[6] V.V. Afsal, S.P. Antony, V.N. Sanjeevan, et al., A new isoform of anti-lipopolysaccharide factor identified from the blue swimmer crab, *Portunus pelagicus*: molecular characteristics and phylogeny, Aquaculture 356–357 (2012) 119–122.
[7] S. Tanaka, T. Nakamura, T. Morita, et al., Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of Limulus coagulation system, Biochem. Biophys. Res. Commun. 105 (1982) 717–723.
[8] S. Tharntada, K. Somboonwiwat, V. Rimphanitchayakit, et al., Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci, Fish Shellfish Immunol. 24 (2008) 46–54.
[9] S.P. Antony, R. Philip, V. Joseph, et al., Anti-lipopolysaccharide factor and crustin-III, the anti-white spot virus peptides in *Penaeus monodon*: control of viral infection by up-regulation, Aquaculture 319 (2011) 11–17.
[10] C. Li, J. Zhao, L. Song, et al., Molecular cloning, genomic organization and functional analysis of an anti-lipopolysaccharide factor from Chinese mitten crab *Eriocheir sinensis*, Dev. Comp. Immunol. 32 (2008) 784–794.
[11] T. Sakai, Crabs of Japan and the Adjacent Seas, Crabs of Japan & the Adjacent Seas, 1976.
[12] Y. Sun, Y. Yan, J. Sun, Larval development of the swimming crab, *Portunus trituberculatus*, J. Fish. China 8 (1984) 219–226.
[13] S.Y. Lee, K. Söderhäll, Early events in crustacean innate immunity, Fish Shellfish Immunol. 12 (2002) 421–437.
[14] P. Chen, J. Li, B. Gao, et al., cDNA cloning and characterization of peroxiredoxin gene from the swimming crab *Portunus trituberculatus*, Aquaculture 322–323 (2011) 10–15.
[15] Z. Cui, C. Song, Y. Liu, et al., Crustins from eyestalk cDNA library of swimming crab *Portunus trituberculatus*: molecular characterization, genomic organization and expression analysis, Fish Shellfish Immunol. 33 (2012) 937–945.
[16] S. Wang, Z. Cui, Y. Liu, et al., The first kazal-type serine proteinase inhibitor in the swimming crab *Portunus trituberculatus* involved in immune response to bacteria and fungi, Aquaculture 356–357 (2012) 55–60.
[17] Y. Liu, Z. Cui, W. Luan, 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.
[18] Y. Liu, Z. Cui, X. Li, et al., A newly identified anti-lipopolysaccharide factor from the swimming crab *Portunus trituberculatus* with broad spectrum antimicrobial activity, Fish Shellfish Immunol. 34 (2013) 463–470.
[19] Y. Liu, Z. Cui, X. Li, et al., A new anti-lipopolysaccharide factor isoform (PtALF4) from the swimming crab *Portunus trituberculatus* exhibited structural and functional diversity of ALFs, Fish Shellfish Immunol. 32 (2012) 724–731.
[20] Y. Liu, Z. Cui, X. Li, et al., Molecular cloning, expression pattern and antimicrobial activity of a new isoform of anti-lipopolysaccharide factor from the swimming crab *Portunus trituberculatus*, Fish Shellfish Immunol. 33 (2012) 85–91.
[21] Z.B. Yu, C.K. Mu, W.W. Song, et al., Screening of genes related to ovarian development in the swimming crab, *Portunus trituberculatus*, by suppression subtractive hybridization, Genet. Mol. Res. 14 (2015) 18675–18686.
[22] S. Kumar, M. Nei, J. Dudley, et al., MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences, Briefings Bioinf. 9 (2008) 299–306.
[23] 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.
[24] P. Casteels, C. Ampe, F. Jacobs, et al., Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*), J. Biol. Chem. 268 (1993) 7044–7054.
[25] A. Hoess, S. Watson, G.R. Siber, R. Liddington, Crystal structure of an endotoxin-neutralizing protein from the horseshoe crab, Limulus anti-LPS factor, at 1.5 A resolution, EMBO J. 12 (1993) 3351–3356.
[26] Y. Yang, H. Boze, P. Chemardin, et al., NMR structure of rALF-Pm3, an anti-lipopolysaccharide factor from shrimp: model of the possible lipid A-binding site, Biopolymers 91 (2009) 207–220.
[27] J. Wismar, T. Löffler, N. Habtemichael, et al., The Drosophila melanogaster tumor suppressor gene lethal (3) malignant brain tumor encodes a proline-rich protein with a novel zinc finger, Mech. Dev. 53 (1995) 141–154.
[28] R. Bonasio, E. Lecona, D. Reinberg, MBT domain proteins in development and disease, Semin. Cell Dev. Biol. 21 (2010) 221–230.
[29] C. Imjongjirak, P. Amparyup, A. Tassanakajon, Molecular cloning, genomic organization and antibacterial activity of a second isoform of antilipopolysaccharide factor (ALF) from the mud crab, *Scylla paramamosain*, Fish Shellfish Immunol. 30 (2011) 58–66.
[30] J. Aketagawa, T. Miyata, S. Ohtsubo, et al., Primary structure of limulus anticoagulant anti-lipopolysaccharide factor, J. Biol. Chem. 261 (1986) 7357–7365.
[31] C.C. Liu, C.P. Chung, C.Y. Lin, et al., Function of an anti-lipopolysaccharide factor (ALF) isoform isolated from the hemocytes of the giant freshwater prawn

Macrobrachium rosenbergii in protecting against bacterial infection, J. Invertebr. Pathol. 116 (2014) 1–7.
[32] C. Sun, W.T. Xu, H.W. Zhang, et al., An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities in vitro and in vivo, Fish Shellfish Immunol. 30 (2011) 295–303.
[33] Y. Zhang, L. Wang, L. Wang, et al., The second anti-lipopolysaccharide factor (EsALF-2) with antimicrobial activity from *Eriocheir sinensis*, Dev. Comp. Immunol. 34 (2010) 945–952.
[34] Q. Ren, Z. Zhang, X.C. Li, et al., Three different anti-lipopolysaccharide factors identified from giant freshwater prawn, *Macrobrachium rosenbergii*, Fish Shellfish Immunol. 33 (2012) 766–774.
[35] X. Li, Z. Cui, Y. Liu, et al., Transcriptome analysis and discovery of genes involved in immune pathways from hepatopancreas of microbial challenged mitten crab *Eriocheir sinensis*, PloS One 8 (2013) e68233.