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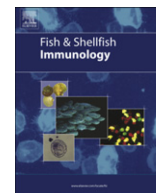

Characterization of two isoforms of antiliopolysacchride factors (Sp-ALFs) from the mud crab *Scylla paramamosain*

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A B S T R A C T

In the previous study of the mud crab (*Scylla paramamosain*) hemocyte proteins, which interacted with a bacterium, *Vibrio parahaemolyticus*, a protein known as antilipopolysaccharide factor (Sp-ALF) was isolated in addition to a serine proteinase homolog (Sp-SPH) protein. In the present study, we further reported the characterization of two isoforms of the mud crab ALF – Sp-ALFs genes (designated as Sp-ALF1 and Sp-ALF2, respectively) based on our previous result. The Sp-ALF1 and Sp-ALF2 cDNA contained 1070 bp and 731 bp, respectively, with 123 deduced amino acid residues. Alignment of deduced amino acid sequences showed that Sp-ALFs possessed high identity with other known ALFs from crustaceans and exhibited an overall similarity of 57.7% to those of ALFs compared. Phylogenetic tree analysis revealed a clear group of each species and also suggested that ALFs from *Scylla* genus and those from *Portunus* genus were closely related. Tissue distribution analysis in adult crab implied that both Sp-ALF1 and Sp-ALF2 were mainly expressed in hemocytes. The mRNA transcripts were also found in embryo (I, II, III and V), zoea-I and juvenile crab, but were rarely observed in the megalopa stage. To further identify the biological activity of Sp-ALFs, recombinant proteins (rSp-ALFs: designated as rSp-ALF1 and rSp-ALF2, respectively) were obtained by expression in *Pichia pastris*, and the synthetic peptide fragments (sSp-ALFs: designated as sSp-ALF1 and sSp-ALF2, respectively) including the putative LPS binding loop were also prepared for antimicrobial test. The results indicated that both rSp-ALFs and sSp-ALFs were highly effective against most of the Gram-positive bacteria and Gram-negative bacteria tested. In contrast to cecropin P1, a membrane integrity assay revealed that Sp-ALFs did not affect the *Escherichia coli* by disruption of membrane integrity. Additionally, the recombinant Sp-ALFs proteins exhibited strong antiviral activity against an important aquaculture pathogen, white spot syndrome virus, in crustaceans. Taken together, these data suggested that Sp-ALFs might play a key role in immune defense against microbial infection in the mud crab *S. paramamosain*.

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1. Introduction

The mud crab *Scylla paramamosain*, a commercially important crustacean, is widely distributed along the coast line of southern China and Pacific area. At present, bacterial infections are the main causes of crab diseases constraint on the crab farming in China [1]. As we know, invertebrates including the crab only have innate immune defense in which non-self recognition of invading microbes is dependent on the pattern-recognition of pathogen-associated molecular patterns (PAMPs) derived from microbial cell-wall components. This recognition employs certain cell-surface receptors like Tolls and Toll-like receptors (TLRs) to serve as conserved signaling pathway in innate immune system both in invertebrates and mammals. Many immune related receptors have been characterized from the hemocytes, which are the primary effectors involved in host defense including phagocytosis, encapsulation, melanization, coagulation and releasing of antimicrobial peptides (AMPs) in invertebrates. It is well known that AMPs, as the most common effectors affecting the invading microorganisms in crustaceans, function as the front line of host defense against microbe infection with a broad spectra of antimicrobial activity to kill Gram-positive and Gram-negative bacteria, fungi, parasites and viruses [2].

To date, many AMPs, such as carcinin [3], crustin [4], anti-lipopolysaccharide factor [5,6] have been described from different crab species. However, few studies have been carried out by direct

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interaction between crab hemocyte components and the pathogenic bacterium, which would provide more details concerning the host–pathogen interactions. In our previous study, an ALF protein was isolated together with a serine proteinase homolog by directly incubating a pathogenic bacterium *Vibrio parahaemolyticus* with the hemocyte lysates from the mud crab [7]. ALF, a well known cationic protein originally identified from the horseshoe crab *Limulus polyphemus*, is capable of binding and neutralizing lipopolysaccharide (LPS) [8,9]. Later, ALF was found to be widely distributed in crustaceans including shrimp [10,11], crab [7] and crayfish [12]. Recently, several studies, regarding to molecular level, have described the gene cloning and organization, expression analysis and functional studies of ALF in various crustaceans [10,13–15]. For example, analysis of ALF genomic organization was carried out in different species including shrimp and crab which may be useful for our better understanding of the control of gene expression with microbial infections [5,11]. Lately, the structure of a shrimp ALF-ALFPm3 was determined with three α-helices packed against a four-stranded β-sheet. And rALFPm3 could bind to both LPS and lipid A [16]. Importantly, the involvement of ALFs in crustacean defense have been approached with both in vivo [12,17] and in vitro [18] antimicrobial activity investigations. For instance, it was found that ALF played a key role in the resistance to both bacterial [17] and viral [12] infection. Meanwhile, the synthetic peptides including part of the ALF fragment exhibited antimicrobial activity against multiple Gram-positive bacterium, Gram-negative bacteria, fungi and viruses [19]. In particular, the shrimp ALFs have been suggested to be involved in protecting animals from bacterial infection [17]. Both the crayfish [12] and the shrimp ALFs [20] were also found to interfere with white syndrome spot virus (WSSV) propagation both in vivo and in vitro. These data together imply that ALFs could act against microbial infection with a broad spectrum in crustaceans. Hence, further functional identification of ALF in the mud crab in terms of efficiently antimicrobial property will bring interesting insights into the disease control in the crab farming.

To characterize the molecules directly interacting with invading microbes, the "bacterial-pellet" methods have been successful used in isolation of a serine proteinase homolog (Sp-SPH) molecules [7]. With this technique, proteins interacting with a bacterium, *V. parahaemolyticus*, were isolated including Sp-ALF protein in addition to the Sp-SPH molecule. The present study, in which the Sp-ALF was further characterized, revealed that both recombinant proteins and synthesized partial peptides of Sp-ALFs inhibited the growth of a panel of Gram-positive and Gram-negative bacteria. A membrane integrity assay implied that Sp-ALFs affected the *Escherichia coli* without the disruption of the bacterial membrane. Further, Sp-ALFs also interfered with WSSV propagation in crayfish haemotopoietic tissue (Hpt) cell cultures. The results reported here together indicated that Sp-ALFs may play a critical role in the immune defense against microbial infection in the mud crab *S. paramamosain*.

2. Matherial and methods

2.1. Full-length cDNA cloning of Sp-ALF1 and Sp-ALF2 genes

To isolate the full-length cDNA of Sp-ALF gene, 5′-RACE and 3′-RACE were carried out. Total RNA from normal crab hemocytes were prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA using the the SMART RACE cDNA Amplification kit (Clontech). Degenerate primers (5′-TGGTGYCCNGGNTGGGCN-CCNTT-3′ and 5′-AANGGNGCCCANCCNGGRCACCA-3′ for 3′-RACE and 5′-RACE, respectively) were designed based on one of the peptide fragments (WCPGWAPF) determined by LTQ-MOLDI-TOF/TOF-MS [7]. The RACE PCR was performed as follows: 94 °C for 4 min; 35 cycles of 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 1 min; and with a final extension at 72 °C for 7 min. The expected fragments were then extracted from agarose gel and ligated into pMD18-T vector (Takara) followed by screening of recombinant clones with bacterial colony PCR. The selected positive clones were sequenced at Shanghai Invitrogen Life Technologies (China). The resulting sequences were then verified and subjected to blast and cluster analysis. Two ALF isoforms (designated as Sp-ALF1 and Sp-ALF2) were then obtained.

2.2. The transcripts distribution of Sp-ALF1 and Sp-ALF2 in various tissues and different development stages of the mud crab

Hemolymph of three normal mud crabs was taken separately as previously described [7], other tissues including brain, eye stalk, gills, heart, hepatopancreas, mid-gut gland, muscle, two sections of reproductive system: GO1 (anterior section: testis, anterior vas deferens, seminal vesicle, posterior vas deferens), GO2 (posterior section: ejaculatory duct, posterior ejaculatory duct, penis), stomach, subcuticular epidermis, and thoracic ganglion mass were also dissected and prepared for total RNA isolation and cDNA synthesis as described above. To determine the mRNA transcripts Sp-ALFs in the different development stages of the crab, the whole embryo (I, II, III and V), zoea (I), and megalopa were used for total RNA isolation. Hemolymph from juvenile (10 ± 2 g, and 35 ± 5 g) and adult (200 ± 50 g) crab were also prepared as previously described [7]. Quantitative real-time PCR was performed in a reaction mixture of 20 μl containing 0.5 ng of total transcribed cDNA, 5 pmol of each gene-specific primer (as listed in Table 1: qALF1+, qALF1−; qALF2+, qALF2−; 18s+, 18s−) and 10 μl of Power SYBR Green PCR Master Mix (Roche). The standard cycling conditions were as 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Data of raw relative quantification were calculated using 7500 system SDS software version 1.3.1.21 and the 18s−Ribosomal gene was employed as the internal standard. ANOVA comparison tests were employed for statistical analysis by SPSS software (version 11.5). Values were considered to be significant as $P < 0.05$.

2.3. Recombinant expression of Sp-ALF1 & Sp-ALF2 in the yeast Pichia pastoris

To characterize the biological activities of Sp-ALF1 and Sp-ALF2, they were both expressed in the yeast *P. pastoris*. The recombinant vector construction and secreted expression were carried out. To obtain the mature peptides of Sp-ALF1 and Sp-ALF2, the cDNA fragments were amplified using specific

Table 1
Primers used in the present study.

Primer	Nucleotide sequence (5′–3′)
eALF1-F	CGTACGTACACCATCATCATCATCATCAGTATGAAACTCTGATAG
eALF1-R	AACCTAGGTTAATTATTCACCCACACCGTAG
eALF2-F	CGTACGTACACCATCATCATCATCATCAGTATGAAGCTCTGGTAGC
eALF2-R	AACCTAGGTTACCCCTTCAGCCAGGCGGCC
5′AOX1	GACTGGTTCCAATTGACAAGC
3′AOX1	GCAAATGGCATTCTGACATCC
Sp18S-F	CAGACAAATCGCTCCACCAAC
Sp18S-R	GACTCAACACGGGGAACCTCA
qSp-ALF1-F	AGGAGCTAGTGAAGGCAATGGA
qSp-ALF1-R	AAAGGCAGTGACGGTGGAGA
qSp-ALF2-F	GGTGAGGCAAGTGATGCAC
qSp-ALF2-R	AAACATCCATTACAGGTCAGACAC
IE1-F	CTGGCACAACAACAGACCCTACC
IE1-R	GGCTAGCGAAGTAAAATATCCCCC
CqGAPDH-F	AATGCTTCTTGCACCACCAAC
CqGAPDH-R	AGGTCTTGCTCAGCTGGATACC

primers eALF1+ paired with eALF1−, and eALF2+ paired with eALF2− as listed in Table 1, both primer pairs were bared with Sna BI and Not I sites in forward and reverse primers, respectively. The PCR products were gel purified and digested with the restriction enzymes before cloned in-frame into Sna BI/NotI sites of the *Pichia* expression vector pPIC9K (Invitrogen). The recombinant plasmids pPIC9K/Sp-ALF1 and pPIC9K/Sp-ALF2 were transformed into *E. coli* DH5α and varified by commercial nucleotide sequencing (Invitrogen, China). After linearization by Sal I, 1 μg of each recombinant was transformed into competent *P. pastoris* by electroporation as recommended by manufacturer's instructions (Invitrogen). After spreading and incubation on MD plate at 30 °C for 48 h, positive clones were screened by PCR with primers 5′AOX and 3′AOX before subjected to recombinant expression induced by 0.5% methonal.

The clones of each recombinant expressing highest amount of recombinant protein were selected for large-scale production. After induction with 0.5% of methonal for 24 h, the protein-containing supernatant was separated from the yeast pellet and dialyzed against phosphate-buffered saline (PBS: 28.5 mM Na_2HPO_4, 21.5 mM NaH_2PO_4, 50 mM NaCl, pH 7.0) before purified by immobilized metal affinity chromatography. The eluted fractions were collected and dialyzed in PBS. The purity of the protein was analysed by SDS-PAGE combined with Coomassie Brilliant Blue staining and the concentration was determined by Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, China). The recombinant protein with a purity of more than 90% was frozen and stored at −80 °C for later use.

2.4. Peptides synthesis of LPS-binding domain of Sp-ALF1 and Sp-ALF2

Linearized LPS-binding domains with two amino acid residues over-hang beyond the conserved cystine residues were synthesized by Shanghai Taishi Biotechnology Co., Ltd (China). The synthetic peptides were derived from positions 53 to 78 (sALF1: HTCHFRRRPKVRKFKLYHEGKFWCPG, sALF2: HTCHIRRRPKFRKF-KLYHE GKFWCPG). Both of the N-terminal residues of these two peptides were blocked by acetylation, and the C-terminal residues were amidated. The synthetic peptides were purified by reverse-phase HPLC with a purity of over 95%.

2.5. Conformation of recombinant expression of Sp-ALF1 and Sp-ALF2 by mass spectrometry analysis

The protein bands corresponding to the recombinant expression were excised from the 15% SDS-PAGE gel and destined twice with 200 μl of 50 mM ammonium bicarbonate (NH_4HCO_3, pH 8.0) mixed with acetonitrile (AcN) (1:1) for 20 min at 37 °C, and then the gel slices were dried completely in SpeedVac. To reduce the protein, 20 μl of 10 mM DTT (in 25 mM ammonium bicarbonate) was added to the gel slice and incubated for 1 h at 56 °C. After that, the protein was alkylated by immediate incubation with 55 mM iodoacetamide (in 25 mM ammonium bicarbonate) for 45 min in the dark. The gel slice was dehydrated gradually with 25 mM NH_4HCO_3 followed by 50 mM NH_4HCO_3 mixed with AcN (1:1), and AcN before drying by evaporation. The gel pieces were re-swelled on the ice for 30 min with 1 μl of 25 mM NH_4HCO_3 containing trypsin (Promega, sequencing grade) at a concentration of 1 ng/μl before adding 10 μl of 25 mM NH_4HCO_3, and then incubated for 12–14 h at 37 °C. Approximately, 1 μl of the supernatant was taken for MALDI-TOF/TOF analysis.

2.6. Antibacterial activity assay

The anti-bacterial activity of rSp-ALFs was tested against five Gram-positive bacteria (*Corynebacterium glutamicum*, *Bacillus subtilis, Micrococcus lysodeikticus*, *Staphylococcus epidermidis*, *M. luteus*) and ten Gram-negative bacteria (*E. coli* mc1061, *Shigella. flexneri, Vibrio harveyi*, Vibrio *fluvialis*, *Vibrio alginolyticus, V. parahaemolyticus, Pseudomonas aeruginosa, P. stutzeri, Aeromonas hydrophila, P. fluorescens*). The minimal growth inhibition concentration (MIC) was determined by the liquid growth inhibition assay. Briefly, the bacteria cultured with mid-logarithmic phase were diluted in 10 mM PBS (pH 7.2–7.4) to $A_{600} = 0.003$ ($3–6 \times 10^5$ CFU/ml). Thirty microliter of this bacteria suspension was mixed with 20 μl of culture media and 50 μl of diluted purified protein to make the assay mixture. After incubation for 24 h at 28 °C, MIC was calculated as the lowest protein concentration yielding visible growth inhibition compared to the negative control. This assay was repeated twice. The minimal bactericidal concentration (MBC) was conducted by inoculating the medium of each well showing no visible growth on an appropriate media, and incubated for 24 h. The MBC was determined as the lowest concentration of protein that prevented any residual colony formation after incubation for 24 h at 37 °C for *E. coli* (mc1061) and *S. epidermidis,* and at 28 °C for the other bacteria tested.

2.7. Membrane integrity assay

To further explore the mechanisms that Sp-ALFs effected on the bacteria, a whole-cell real-time assay using *E. coli* mc1061, constitutively expressing a recombinant luciferase was employed with mildly modification from a previous study [21]. Briefly, 50 μl of MH medium containing 1×10^5 *E. coli* cells (mc1061) and 2 mM D-luciferin potassium salt (Sigma, USA) was mixed with 50 μl dilution of the recombinant proteins under appropriate concentration (1.5 μM and 3 μM) at 25 °C. Luminescence was monitored using a microplate reader (Tecan Infinite, China). One micromole of cecropin P1 (Sigma) was used as the positive control peptide and Argireline (25 μM) (Tash, China) was used as the negative control peptide. To determine the effect of Sp-ALFs on the tested bacteria, as well as exclude the inhibition effect on luciferase activity by the sample processing, additional cecropin P1 (1 μM) was added after 5 min of incubation to the reacting mixtures conducted with Sp-ALFs or Argireline peptide followed by immediate monitoring as mentioned above. All measurements were repeated in triplicates.

2.8. Antiviral activity assay

The hematopoietic tissue (Hpt) cell culture from freshwater crayfish, *Cherax quandricarinatus*, was prepared in a 96-well plate according to the method of Söderhäll et al. as previously described [22]. WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China) and was prepared with absolute quantification via PCR. The effect of rSp-ALFs on WSSV infection was investigated by determining the viral replication in crayfish Hpt cell cultures [12]. The assay was performed by incubation of 10^6 copies of virons/well with two concentrations of purified recombinant proteins (5 and 10 μM) for 30 min at 28 °C. Then the mixture was inoculated into a one-day-old Hpt cell culture (2×10^5/well) and incubated for 12 h at 28 °C followed by total RNA preparation using RNAprep pure Micro Kit (Tiangen, Beijing, China). After DNaseI digestion, 500 ng of total RNA was used for cDNA synthesis with Prime Script™ RT reagent Kit (Takara). The PCR was carried out and transcript of IE1, an immediate early gene of WSSV, was employed to assess the potential

replication of WSSV in crayfish Hpt cells, and a housekeeping gene of Hpt cells, GAPDH, was used as an internal control.

3. Results

3.1. Cloning of the full-length cDNA sequences of Sp-ALF genes

To further determine the full-length cDNA sequence of Sp-ALF gene, degenerate primers were designed according to the partial peptide sequences of WCPGWAPF for 5′ and 3′ RACE PCR as described above [7]. Two ALF isoforms, named as Sp-ALF1 and Sp-ALF2, were then cloned from hemocytes of *S. paramamosian*. As shown in Fig. 1A, the cDNA sequence of Sp-ALF1 contained 1070 bp, including a 97 bp of 5′- untranslated region (UTR), an open reading frame (ORF) of 369 bp, and a 601 bp of 3′-UTR; the cDNA sequence of Sp-ALF2 contained 731 bp, including a 88 bp of 5′-UTR, an ORF of 369 bp, and a 170 bp of 3′-UTR (Fig. 1B). The cDNA sequences of Sp-ALF1 and Sp-ALF2 shared 77.2% similarity. Both Sp-ALFs were predicted to possess a 26 aa signal peptide by SignalP (http://www.cbs.dtu.dk/services/SignalP/) in the N-terminus. The calculated molecular mass of the mature peptide without signal peptide was 11.28 kDa with an estimated isoelectric point (pI) of 10.35 for Sp-ALF1, and 11.18 kDa with an estimated pI of 9.95. The full-length cDNA sequence and their deduced amino acid sequences for Sp-ALF1 and Sp-ALF2 were deposited in the Genbank under accession numbers of JQ069030 and JQ069031, respectively.

The deduced amino acid sequences of Sp-ALF1 and Sp-ALF2 (123 amino acids) gave a high similarity 57.7% to the other ALFs from crustaceans such as swimming crab (*Portunus trituberculatus*), horseshoe crab (*Tachypleus tridentatus*), shrimp (*Penaeus monodon*), crayfish (*Pacifastacus leniusculus*) and Chinese mitten crab (*Eriocheir sinensis*) (Fig. 2A). Especially, Sp-ALF2 showed the same deduced amino acid sequences to ALFSp [5]. Both Sp-ALF1 and Sp-ALF2 contained two conserved cysteine residues and the clustering of positive charges were mainly located within the disulfide loop (55–76 residues). This positive cluster domain was defined as the putative LPS binding site similar to the other ALFs reported from horseshoe crab [5,6], shrimp [16] and crayfish [12]. Phylogenetic analysis of Sp-ALF1 and Sp-ALF2 revealed that these two ALFs were similar to the ALFs from other crustacean species as shown in Fig. 2B. Specifically, it was found that the ALFs from *Scylla* genus and *Portunus* genus were clustered into one group, and those from the crayfish (PlALF), and shrimp (PmALF1, PmALF2 and MjALF2) were closely related.

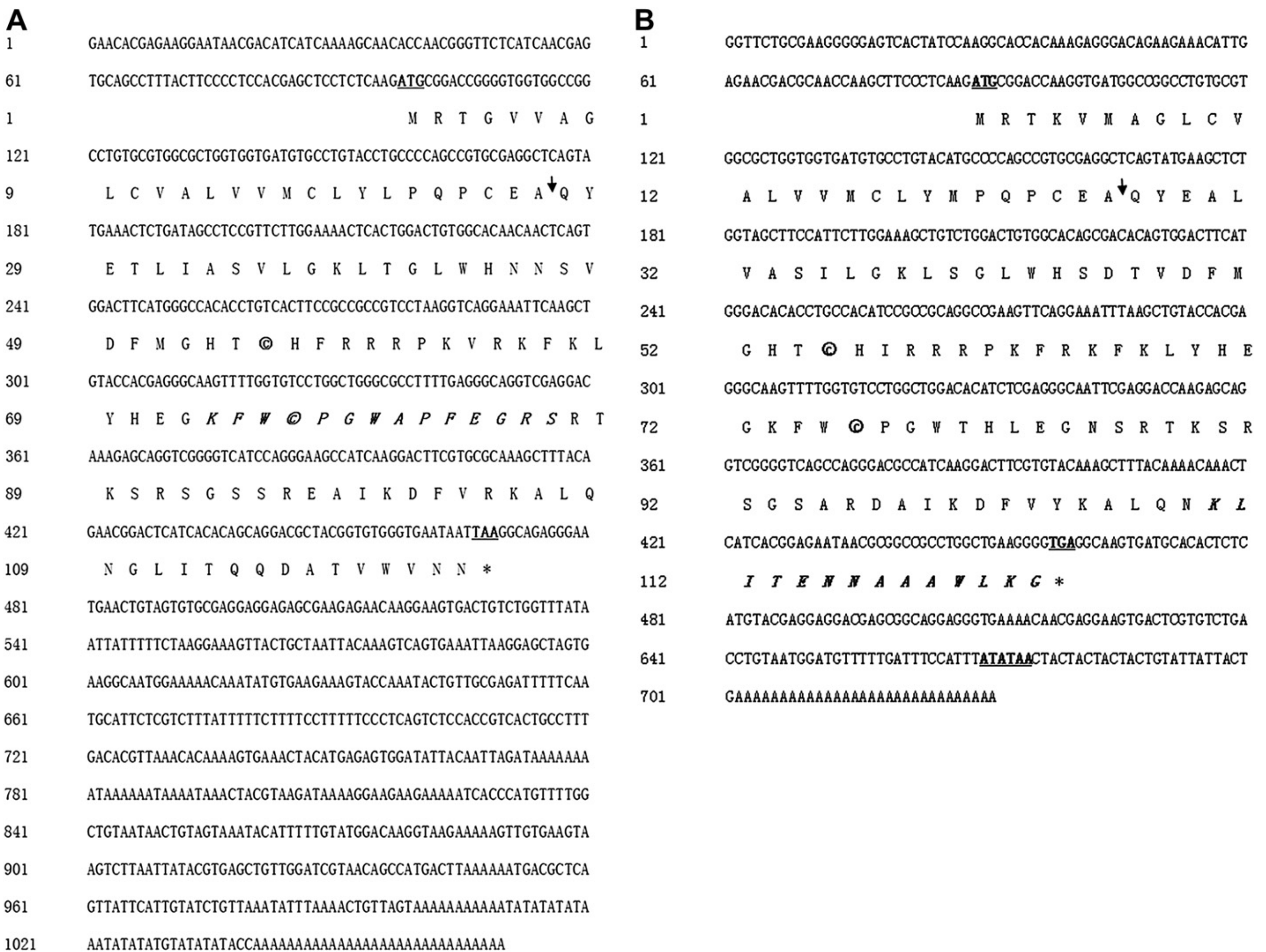

Fig. 1. The full-length cDNA sequences (above) and predicted amino acid sequences (below) of Sp-ALF1 and Sp-ALF2 from the mud crab *S. paramamosain*. Partial amino acid sequences of the different bands determined chemically were in bold and italic. The proposed start codon, stop codon and putative polyadenylation signal were in bold and underlined. Arrow indicated the predicted cleavage site for the signal peptide. The conserved cysteine residues forming the LPS-binding domain were in circles and bold.

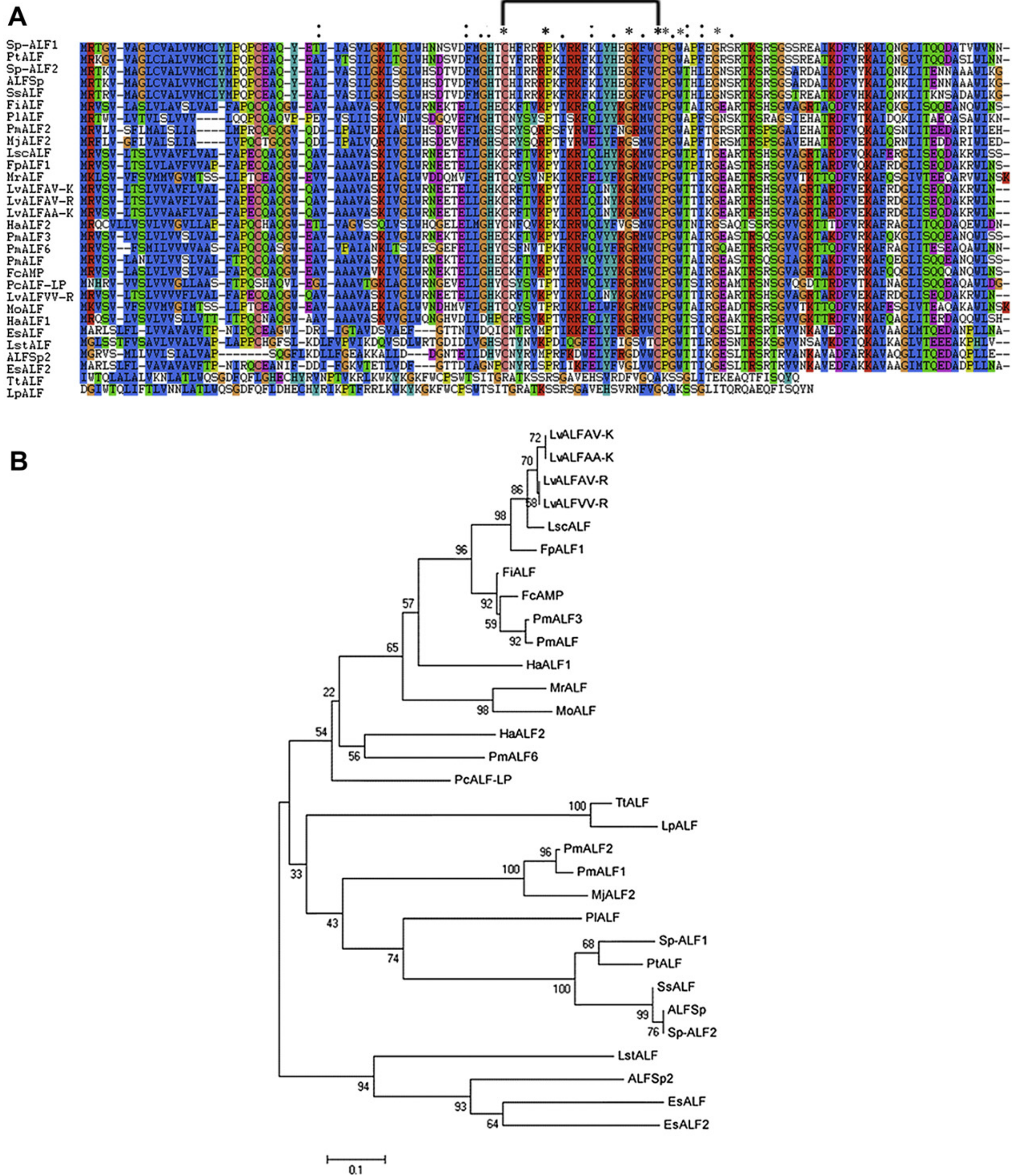

Fig. 2. Multiple alignment of amino acid sequences. (A) And phylogenetic analysis. (B) of Sp-ALFs with other known ALFs. PtALF, *Portunus trituberculatus* (C0KJQ4.2); ALFSp, *Scylla paramamosain* (ABP96981.1); SsALF, *S. serrata* (ADW11095.1); FiALF, *Fenneropenaeus indicus* (ADE27980.1); PlALF, *Pacifastacus leniusculus* (ABQ12866.1); PmALF2, *Penaeus monodon* (ABP73291.1); MjALF2, *Marsupenaeus japonicus* (BAH22585.1); LscALF, *Litopenaeus schmitti* (ABJ90465.1); FpALF1, *Farfantepenaeus paulensis* (ABQ96193.1); MrALF, *Macrobrachium rosenbergii* (ACG60660.2); LvALFAV-K, *L. vannamei* (ACT21197.1); LvALFAV-R, *L. vannamei* (ABB22832.1); LvALFAA-K , *L. vannamei* (ABB22833.1); HaALF2, *Homarus americanus* (ACC94269.1); PmALF3, *P. monodon* (ABP73289.1); PmALF6, *P. monodon* (ADM21460.1); PmALF, *P. monodon* (ADC32520.1); FcAMP, *Fenneropenaeus chinensis* (AAX63831.1); PcALF-LP, *Procambarus clarkii* (ADX60063.1); LvALFVV-R, *Litopenaeus vannamei* (ABB22831.1); MoALF, *M. olfersii* (ABY20736.1); HaALF1, *H. americanus* (ACC94268.1); EsALF, *Eriocheir sinensis* (ABG82027.1); LstALF, *L. stylirostris* (AAY33769.1); ALFSp2, *S. paramamosain* (ADT71677.1); EsALF2, *E. sinensis* (ACY25186.1); TtALF, *Tachypleus tridentatus* (AAK00651.1); LpALF, *Limulus polyphemus* (1307201A). (A) The conserved cysteines forming putative intramolecular disulfide bonds were connected by solid lines. (B) Numbers at the forks indicated percentage bootstrap values: the percentage of times that the particular node occurred in 1000 trees which were generated by bootstrapping the original deduced protein sequences.

3.2. Tissue distribution of Sp-ALF1 and Sp-ALF2 transcripts in the mud crab S. paramamosain

We found that the mRNA expression level of Sp-ALF1 and Sp-ALF2 varied among the samples tested with the transcript distribution of Sp-ALF1 and Sp-ALF2 in various tissues examined by real-time PCR. As shown in Fig. 3A, though the mRNA transcripts of Sp-ALF1 and Sp-ALF2 were both highly expressed in hemocyte, Sp-ALF1 transcript was present in anterior section of reproductive tract with relatively higher expression followed by lower expression in

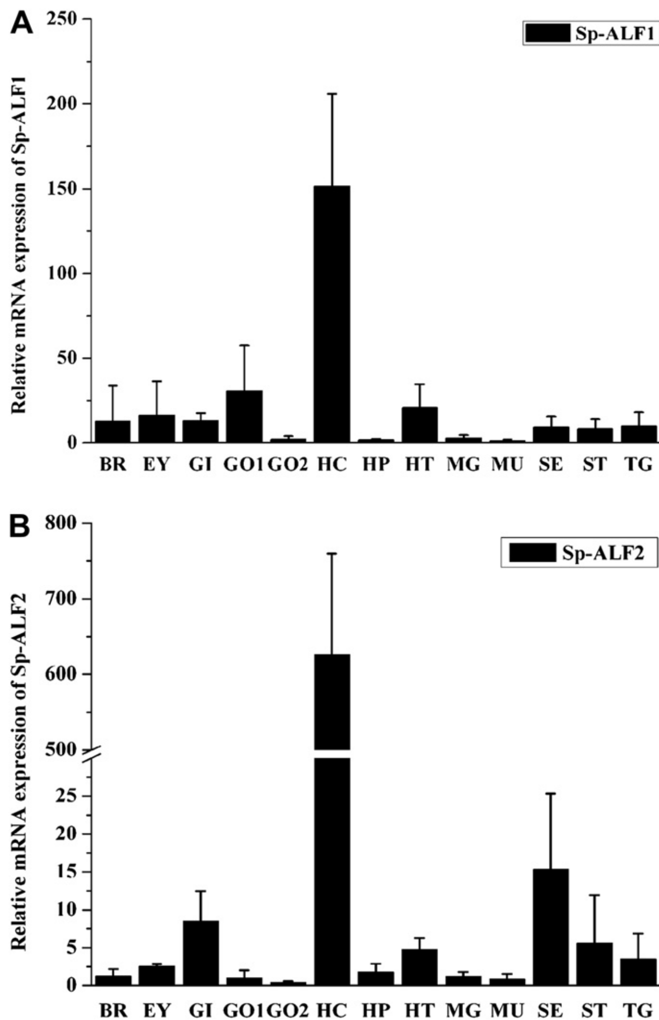

Fig. 3. Distribution of Sp-ALF1. (A) And Sp-ALF2. (B) mRNA transcripts in various tissues of the mud crab *S. paramamosain*. MU: muscle; HE: hemocyte; HP: hepatopancreas; GI: gill; MG: mid-gut; ST: stomach; SE: subcuticular epidermis; HT: heart; TG: thorax ganglion; BR: brain; ES: eye stalk; GO1: anterior section of reproductive tract (testis, anterior vas deferens, seminal vesicle, posterior vas deferens); GO2: posterior section of reproductive tract (ejaculatory duct, posterior ejaculatory duct, penis). The transcript level in muscle was used as a calibrator for relative comparison. The experiment was repeated for three times, and the data represented means of triplicates. Bars indicated mean ± S.E. ($n = 3$).

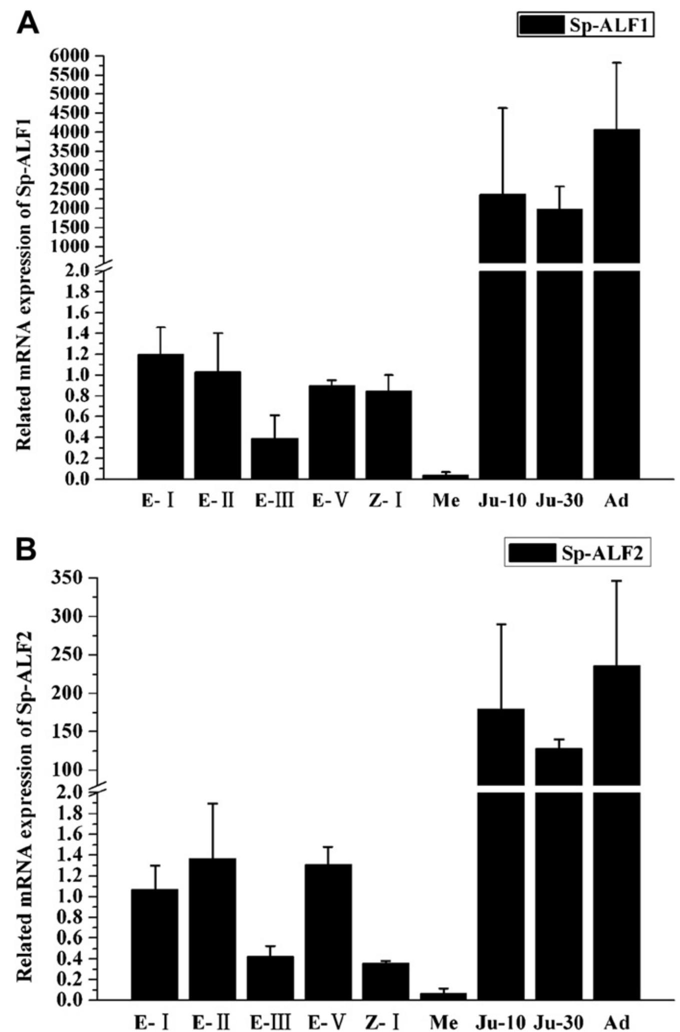

Fig. 4. Determination of Sp-ALF1. (A) And Sp-ALF2 (B) transcripts in different development stages of mud crab *S. paramamosain*. E–I: embryo stage I; E-II: embryo stage II; E-III: embryo stage III; E-V: embryo stage V; Z-I: zoea-I stage; Me: megalops; Ju-10 g: hemocyte from juvenile crab (10 ± 2 g); Ju-30 g: hemocyte from juvenile crab (30 ± 5 g); Ad: hemocyte from adult crab. The experiment was repeated for three times, and the data represented means of triplicates. Bars indicated mean ± S.E. ($n = 3$).

heart, eye stalk, gill and brain, and was hard to be detected in the posterior part of reproductive tract, hepatopancreas, mid-gut and muscle. While Sp-ALF2 was evidently expressed in subcuticular epidermis followed by gill, stomach, thorax ganglion and eye stalk. Additionally, there was little possibility to detect Sp-ALF2 transcript in reproductive tract. Thus, different expression patterns of Sp-ALFs suggested that Sp-ALF1 and Sp-ALF2 might play various roles in the mud crab. Further, we determined the mRNA expression of Sp-ALF1 and Sp-ALF2 in selected developmental stages in which the Sp-ALF1 and Sp-ALF2 exhibited similar expression patterns with the highest expression of both Sp-ALF1 and Sp-ALF2 in the hemocyte and less high expression in the embryo and zoea stages. Sp-ALF1 and Sp-ALF2 were barely detected in the megalopa stage (Fig. 4), suggesting they might play a certain role in embryo stage as well as other developmental stages.

3.3. Recombinant expression of Sp-ALF1 and Sp-ALF2 in yeast

In order to further characterize the Sp-ALFs in terms of biological activity, both mature peptides of Sp-ALFs were recombinantly expressed with the yeast expression vector pPIC9k. After methonal induction for 24 h, high amounts of recombinant Sp-ALF1 and Sp-ALF2 were obtained, respectively. The recombinant proteins secreted in the yeast culture medium were purified with a single-step purification using a Ni-NTA affinity column. As shown in Fig. 5, both of the recombinant proteins showed a major band about 13 kDa, which were in agreement with the calculated molecular mass based on their deduced amino acid sequences including six histidine residues. The rSp-ALF1 and rSp-ALF2 were also confirmed by MALDI-TOF/TOF mass spectrometry analysis in which several peptide fragments corresponding to the deduced protein sequences of Sp-ALF1 and Sp-ALF2 were confirmed. Meanwhile, the recombinant Sp-ALF1 protein showed an additional band of approximately 17 kDa. The mass spectrum analysis revealed this band was also in agreement with the fragment of the deduced amino acids of Sp-ALF1 (Fig. S1). We then analyzed both of Sp-ALF1 and Sp-ALF2 for putative glycosylation site by using NetOGlyc 3.1 Server (http://www.cbs.dtu.dk/services/NetOGlyc/). A putative glycosylated Threonine^{118} was observed in Sp-ALF1, but no glycosylation site was found in Sp-ALF2, suggesting the putative

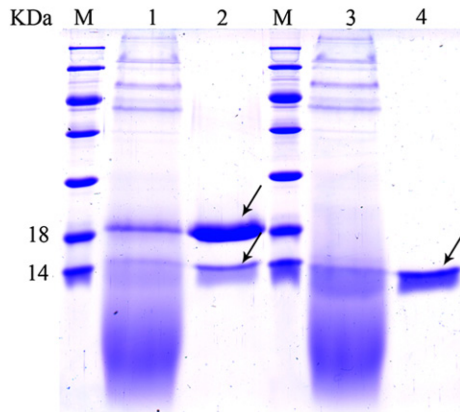

Fig. 5. SDS–PAGE analysis of the purified rSp-ALF1 and rSp-ALF2 with 6 × His tag by Ni^{2+} affinity chromatography. Lane M, molecular weight marker; lane 1, 3, cultured medium from pPIC9K/Sp-ALF1 and pPIC9K/Sp-ALF2 recombinant clones, respectively, induced by methonal before purification; lane 2, purified rSp-ALF1; lane 4, purified rSp-ALF2.

glycosylation might occur in Sp-ALF1 during the recombinant expression in the yeast expression system.

3.4. Bacterial growth inhibitory and bactericidal assays

The antimicrobial and bactericidal activities of the recombinant Sp-ALFs (rSp-ALFs) were determined by MIC and MBC tests, respectively, against a panel of microorganisms including the pathogenic strains like vibrios for crab and shrimp. As shown in Table 2, rSp-ALF1 and rSp-ALF2 exhibited a broad spectrum of anti-bacterial activities toward both Gram-positive bacteria (*C. glutamicum*, *B. subtilis, M. lysodeikticus*, *M. luteus*) and Gram-negative bacteria (*E. coli* mc1061, *Shigella flexneri, V. harveyi, V. alginolyticus, V. parahaemolyticus, P. aeruginosa, P. stutzeri, P. fluorescens*) with MIC values lower than 12.5 μM, as well as a bactericidal effect (MBC < 12.5 uM) except for *V. fluvialis*, *A. hydrophila*, and *S. epidermidis* in which they were not affected with the concentration of lower than 12.5 μM. Notably, rSp-ALF1 was effectively against the growth of Gram-positive bacteria (*B. subtilis, M. luteus*) and Gram-negative bacteria (*E. coli, P. fluorescens, P. stutzeri, V. harveyi, V. alginolyticus, V. parahaemolyticus*) with an MIC value lower than 1.6 μM, and against other bacteria tested (Gram-positive: *C. glutamicum*, *M. lysodeikticus*; Gram-negative: *P. aeruginosa*, *S. flexneri*) with an MIC value lower than 6.2 μM. Compared to that of rSp-ALF1, rSp-ALF2 showed similar anti-bacterial property against the strains tested but with slightly lower anti-bacterial efficiency against some of the bacteria tested. We also studied the antimicrobial activity of the synthetic Sp-ALFs corresponding to a part of the putative LPS binding loop of Sp-ALFs and compared to those of the recombinant rSp-ALFs. As shown in Table 2, both synthetic peptides exhibited stronger or similar activities against all the Gram-positive bacteria (*B. subtilis, C. glutamicum*, *M. luteus*, *M. lysodeikticus*, *S. epidermidis*; MIC ≤ 12.5 μM and MBC ≤ 12.5 μM) and one Gram-negative bacterium (*P. fluorescens*; MIC ≤ 1.6 μM and MBC ≤ 6.2 μM) tested. Generally, the synthetic partial fragments of Sp-ALF peptides were as efficient as their mature peptides against the Gram-positive bacteria tested, which gave lower or similar MIC values. While they were much less effective against some Gram-negative bacteria including *A. hydrophila*, *P. aeruginosa*, *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*. On the other hand, the sSp-ALF2 exhibited slightly stronger anti-bacterial property against the bacteria tested compared to that of sSp-ALF1. Additionally, ineffective activity by recombinant rSp-ALFs was found against the highly pathogenic bacterium, *A. hydrophila* (MIC > 25 μM), which causes serious diseases in aquiculture farming. However, the synthetic peptides especially sSp-ALF2 gave stronger inhibitory activity toward this bacterium with an MIC value lower than 12.5 μM, suggesting that the anti-bacterial activities were different between the recombinant proteins and the synthetic peptides. These results together implied that the Sp-ALFs may function efficiently against bacterial infection in the immune defenses in the mud crab.

3.5. Membrane integrity assay

Several studies have investigated the LPS binding ability of the recombinant ALF [23]. Besides, it was proposed that the bactericidal effect of ALF like ALFPm3 from shrimp was due to its binding activity to components of the bacterial cell-wall [24]. To investigate

Table 2
Summary of minimum inhibitory concentration (MIC, μM) and minimum bactericidal concentration (MBC, μM) of recombinant Sp-ALFs (rSp-ALFs) and synthetic Sp-ALFs (sSp-ALFs) corresponding to a part of the putative LPS binding domain.

Bacteria	CGMCC NO.[a]	rSp-ALF1		rSp-ALF2		sSp-ALF1		sSp-ALF2	
		MIC[c]	MBC[c]	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive bacteria									
Bacillus subtilis	1.108	<1.6	<3.1	<3.1	<6.2	<3.1	<3.1	<1.6	<3.1
Corynebacterium glutamicum	1.1886	<6.2	<12.5	<3.1	<12.5	<1.6	<3.1	<1.6	<1.6
Micrococcus lysodeikticus	1.0634	<6.2	<6.2	<3.1	<6.2	<3.1	<3.1	<1.6	<1.6
M. luteus	1.634	<1.6	<1.6	<1.6	<3.1	<1.6	<1.6	<1.6	<1.6
Staphylococcus epidermidis	1.2429	>12.5	>12.5	>12.5	>12.5	<12.5	<12.5	<12.5	<12.5
Gram-negative bacteria									
Aeromonas hydrophila	1.1801	>25	>25	>25	>25	<25	<25	<12.5	<12.5
E. coli mc1061	ATCC: 25922[b]	<1.6	<3.1	<3.1	<12.5	<12.5	<25	<1.6	<6.2
Pseudomonas aeruginosa	1.0205	<6.2	<6.2	<6.2	<12.5	>25	>25	>12.5	>12.5
P. fluorescens	1.0032	<1.6	<3.1	<3.1	<3.1	<1.6	<6.2	<1.6	<1.6
P. stutzeri	1.1803	<1.6	<1.6	<3.1	<3.1	<3.1	<12.5	<1.6	<1.6
Shigella flexneri	1.1868	<3.1	<3.1	<3.1	<3.1	<1.6	<25	<1.6	<3.1
Vibrio alginolyticus	1.1833	<1.6	<3.1	<3.1	<3.1	<25	>25	>12.5	>12.5
V. fluvialis	1.1609	>12.5	>12.5	>12.5	>12.5	>25	>25	>12.5	>12.5
V. harveyi	1.1593	<1.6	<1.6	<3.1	<6.2	<12.5	>12.5	>12.5	>12.5
V. parahaemolyticus	1.1615	<1.6	<3.1	<3.1	<3.1	>25	>25	>25	>25

[a] CGMCC NO. China General Microbiological Culture Collection Number.
[b] ATCC: American Type Culture Collection.
[c] All the concentrations showed in this table were in μM. The values of MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) were expressed as the lowest concentration yielding no detectable microbial growth or that killed more than 99.9% of microorganisms ($n = 3$).

whether Sp-ALFs affect the integrity of bacterial membrane, in terms of bactericidal or bacteriostatic effect, a membrane integrity assay detecting the release of luciferase by a transgenetic *E. coli* cells (mc1061) was measured as reported [25]. Mid-logarithmic phase *E. coli* expressing firefly luciferase were incubated with 2 mM D-luciferin at pH 7.4. Recombinant Sp-ALF1 or Sp-ALF2 was added to a final concentration of 1.5 μM and 3 μM (according to the MIC results), respectively, and changes in light emission were monitored. Addition of 1 μM cecropin P1 to the *E. coli* served as a positive control as it inserted pores into membranes resulting in significant luciferase release. As determined by the luciferase flux signal a strong peak of light emission typical for the membrane disruption by AMP [26] was found in cecropin P1 treated *E. coli*. However, no significant increase of light emission was observed after addition of neither Sp-ALF1 nor Sp-ALF2 in comparison to the cecropin P1 treatment and the negative controls including Argireline treatment or non-protein samples (Fig. 6). To exclude the possibility that the peptides inhibited luciferase activity, cecropin P1 was added after 5 min of incubation to the reactions conducted with rSp-ALFs or water. As shown in Fig. S2, no significant light emission was acquired after the second addition of cecropin P1 to the pretreated *E. coli* with Sp-ALFs. This result implied that rSp-ALF1 and rSp-ALF2 were unlikely to enhance the permeability of the bacterial membrane.

3.6. Inhibition of WSSV infection by Sp-ALFs in crayfish Hpt cell cultures

Given the antiviral activity of crayfish *Pl*ALF [12] and shrimp ALF*Pm*3 [20], we then determined if the mud crab ALFs also interfered with WSSV propagation in crayfish Hpt cell cultures. To test whether the Sp-ALF protein could directly neutralize the virus, the WSSV was co-incubated with the rSp-ALFs for 30 min before its inoculation into crayfish Hpt cell cultures. As shown in Fig. 7, a significant reduction of IE1 transcript was detected when the crayfish Hpt cells incubated with WSSV pretreated with 5 or 10 μM of rSp-ALFs in a concentration dependent manner. Meanwhile, different inhibitory efficiency of WSSV propagation was observed

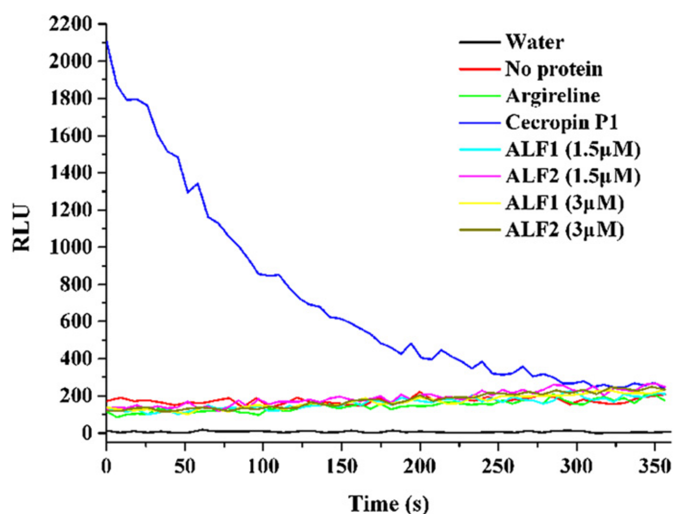

Fig. 6. Effect of rSp-ALF1 and rSp-ALF2 on the bacterial membrane integrity. The treatment of the bacterial membranes by membranolytic peptide led to catalysis of D-luciferin by luciferase and resulted in light emission. Light emission kinetics of the luciferase expressing *E. coli* (mc1061) was then recorded after treatment with AMPs. The light emission from different treatments was recorded as follows: rSp-ALFs (1.5 μM and 3 μM), Argireline (3 μM), and cecropin P1(1 μM). Related light unit (RLU) was detected as time developed.

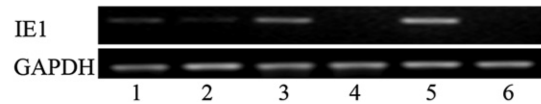

Fig. 7. Antiviral effect of rSp-ALF1 and rSp-ALF2 on WSSV propagation in crayfish Hpt cell cultures. Semi-quantitative RT-PCR was used to detect IE1 transcript to evaluate the anti-WSSV effect of the recombinant Sp-ALFs with GAPDH as an internal control. Lane1-2, Hpt cells inoculated with WSSV mixture containing rSp-ALF1 (5, 10 μM) for 3 h; Lanes 3–4, Hpt cells treated with WSSV pretreated with rSp-ALF2 (5, 10 μM) for 3 h; Lane 5, Hpt cells inoculated with equal amount of WSSV pretreated with sterilized water for 3 h as a positive control; Lane 6, Hpt cells without WSSV inoculation as a negative control. All the treatments were incubated for 30 min before adding to the crayfish Hpt cell cultures.

between rSp-ALF1 and rSp-ALF2, in which high concentration of rSP-ALF2 exhibited strongly inhibitory efficiency compared to that of rSP-ALF1. This result clearly indicated that the Sp-ALFs from mud crab were also effective on the inhibition of an important marine virus, WSSV, replication in crayfish Hpt cell cultures but with different efficiencies, suggesting the antiviral function of various ALFs from the mud crab.

4. Discussion

In the previous study, we used a crab pathogenic bacterium *V. parahaemolyticus* as "bacterial beads" to isolate the immune factors directly from the hemocyte lysate of mud crab. The binding proteins were further characterized by LTQ mass spectrum to be an Sp-SPH protein and an ALF protein [7]. So far, a number of ALFs genes have been identified with obviously conserved character as antimicrobial property, indicating the likely important role in immune defense in crustaceans. However, the function of Sp-ALFs in crab immunity is not yet clearly understood. According to the structure analysis of ALFs [27], the ALFs enclosed a potential LPS-binding site which could form a hairpin loop. This loop contained a number of conserved amino acid residues. Most of the conserved amino acids were hydrophobic and belonged to the hydrophobic core of ALF structures [16]. In the present study, the full-length cDNA of two ALFs gene isoforms from mud crab were successfully identified based on the deduced protein sequences described above. Similar LPS binding domain was also found in Sp-ALF1 and Sp-ALF2. Comparative analysis of Sp-ALF1 and Sp-ALF2 indicated highly conserved LPS binding domain as reported in the other ALFs, suggesting the conservation of LPS binding activity. Further, amino acid sequences alignment indicated Sp-ALFs shared high identity with other reported ALFs from crustaceans including mud crab (*Scylla serrata*), swimming crab, crayfish, shrimp and horseshoe crab. The phylogenetic tree analysis showed that the Sp-ALF1 and Sp-ALF2 were clustered separately from those of shrimp and horseshoe crab, which were grouped with marine crabs including *Scylla* and *Portunus* genus.

It is well known that the immune defense molecules are mainly stored in the hemocytes or secreted into plasma from the hemocytes in crustaceans [28]. Generally, three categories of the hemocytes are defined as: hyaline cells, semigranular cells and large granular cells from decapod crustaceans [29]. As previously isolated from crab hemocyte lysate with directly binding affinity to bacterium, both Sp-ALF1 and Sp-ALF2 also exhibited abundant transcripts in hemocytes but with lower expression in hepatopancreas, implying that hemocyte was the dominant site for generation of Sp-ALFs in the mud crab as well as other crab species [6,30,31]. This expression profile was similar to a shrimp MjALF1 in which the highest expression was found in the hemocyte [13]. ALF was also suggested to be expressed in different hemocyte populations in shrimp [32]. While in signal crayfish,

*Pl*ALF was abundantly expressed in both semigranular cells and granular cells [12]. Hence, these cells may perform certain roles in terms of immune defense toward invading pathogens in crustaceans. It is clear that ALF transcription, although tissue specific, is present in multiple organs, in addition to hemocytes, and they might give systemic protection toward pathogens [17]. Here, transcript of Sp-ALF1 but not Sp-ALF2 was also observed in mud crab reproductive tract. A crab AMP, named as scygonadin, has been described to be highly expressed in the reproductive tract of male *Scylla* crab with a broad anti-bacterial spectrum, implying its possible role in the reproductive immunity [33]. Regarding the antimicrobial function and presentation in the crab reproductive tract, Sp-ALF1 might have complementary activity to those shown by the scygonadin. Hence, it is worthy to further study both Sp-ALFs and scygonadin regarding their temporal expression and localization at different developmental stages as well as responses to microbial infection. These data provided the evidence for multifunction of various ALFs in crusteans. Thus, the study for elucidating the anti-bacterial infection of the hemocyte ALFs is necessary for future better control or prevention of the bacterial caused diseases in the mud crab.

The cationic AMPs were proved to bind to the anionic microbial components including the lipid A site of the LPS from Gram-negative bacteria and the lipoteichoic acid (LTA) from Gram-positive bacteria [23,34]. ALF proteins or ALF-derived peptides exhibited binding activity to bacterial LPS as well. In addition to its direct antimicrobial property to bacteria, ALF has been suggested as a key immune effector due to its LPS-binding role to many responses such as inhibition of inflammatory reaction or anticoagulant function in crustaceans [13,35,36]. Particularly, the expression of ALF in crustaceans was likely to be up-regulated post microbial challenge [12–14,31,37]. Several studies revealed that ALF was involved in the immune responses to eliminate invasive pathogens [13,38]. As important antimicrobial peptides, ALFs gave a broad spectrum against Gram-negative bacteria, Gram-positive bacteria, fungi, and viruses [39]. To characterize the biological features of Sp-ALFs, we prepared recombinant Sp-ALFs in *P. pastoris*, which is appropriate for the production of large amount of functional molecules. We found the recombinant Sp-ALFs affected all Gram-positive bacteria strains and most of Gram-negative bacteria strains tested. As the most pathogenic bacteria present in marine environment, Gram-negative bacteria and Vibrionaceae genera have been caused serious disease in marine crustaceans. It is then necessary to elucidate the function of ALF against these pathogenic microorganisms. On the other hand, the synthetic peptide enclosing a putative LPS binding site described for *Limulus* ALF [27] exhibited a strong antimicrobial property against several Gram-positive and Gram-negative bacteria [5,13,18]. Similar synthetic peptide fragments according to ALF sequences also gave potent antiviral property in crustaceans [5,12,18,20]. In the present study, both the synthetic rSp-ALF1 and rSp-ALF2 peptides, composed with 26 amino acid residues with the predicated LPS binding loop, showed clear antimicrobial activity against Gram-positive and Gram-negative bacteria. The antimicrobial activity of both sSp-ALF1 and sSp-ALF2 showed quite stronger effect to, if compared to those of recombinant rSp-ALFs, all the tested Gram-positive bacteria (*B. subtilis*, *C. glutamicum*, *M. luteus*, *M. lysodeikticus* and *S. epidermidis*) and one Gram-negative bacterium (*P. fluorescens*) tested with lower MIC values. In contrast, highly weaker antimicrobial activity of the synthesized sSp-ALF1 was observed for this peptide fragment against most of the Gram-negative bacteria tested with the exception of *A. hydrophila*, and *P. fluorescens*. Regarding the anti-bacterial efficiency of synthesized peptide fragments, the synthesized sSp-ALF2 provided higher antimicrobial efficiency against most of the tested Gram-positive bacteria (*B. subtilis, C. glutamicum*, *M. luteus* and *M. lysodeikticus*) and Gram-negative bacteria (*A. hydrophila*, *E. coli* mc1061, *P. aeruginosa*, *P. stutzeri*, *S. flexneri* and *V. fluvialis*) compared to that of sSp-ALF1. These data were useful for the peptide designing in the applied use for mud crab farming.

AMPs, like cecropin P1, could affect bacterium by disruption of its membrane integrity. Previously, rSsALF was reported to permeabilize artificial phospholipids membrane by using a calcein enclosed liposome model [30]. Here, we found that membrane pore formation was unlikely to be the cause for the anti-bacterial activity of both Sp-ALF1 and Sp-ALF2. As previously reported that SpStrongylocins [25] and hepcidin [40] did not act on the bacterium by disruption of membrane integrity. But it could not be excluded that Sp-ALF1 and Sp-ALF2 might interfere with bacterial membrane integrity under other assay conditions except the experimental design here or that pores were generated which only allowed the passage of molecules smaller than D-luciferin [21]. Hence, the mechanism of inhibition on bacteria by Sp-ALFs should be further studied.

The AMPs such as tacheplesin, polyphemusin, and mytilin from crustaceans possess clearly antiviral property against various viruses [41–43]. In addition to its role in anti-bacterial activity, both *Pl*ALF and rALFPm3 from crayfish and shrimp, respectively, exhibited strongly antiviral activity against WSSV [12,20]. WSSV is one of the most serious viral pathogens in shrimp farming and also is infecting many other species of crustaceans including the genus *Scylla* crab [44]. Further, *Scylla* crab is not only susceptible to WSSV, but also is a common viral carrier in the farming ponds. So far, several ALFs have been identified from the crab species like Sp-ALF [7], ALFSp/ALFSp2 [5,6], PtesALF1-3 [45], and EsALF [31,46]. Little is known about the crab ALF against WSSV infection neither in vivo nor in vitro. Importantly, an obviously inhibition on WSSV were also observed in rSp-ALFs in the present study. This was the first implication in vitro that the crab ALF was effective against the WSSV. The mechanism of ALF against WSSV infection is unclear. While, the rALFPm3 was suggested in some way to prevent WSSV efficiently from binding and/or entering into the crayfish cells [20]. Pretreatment of Hpt cells with PlALF, the synthetic crayfish ALF peptide, corresponding to the putative LPS-binding site, could protect Hpt cell cultures against WSSV infection but not shrimp rALFPm3. The mechanism of protective (or prophylactic) effect of ALF on Hpt cell cultures against viral infection needs further studies. Likely, the crustacean ALFs could be a highly potent immune effector in protecting the animals against WSSV infection, implying its promising use for antiviral control in aquculture. In order to determine the importance of Sp-ALFs in the antiviral protection of crab to viral challenges, the protective experiments by feeding the animals with Sp-ALFs as additives should be further investigated.

In summary, we have successfully identified the full-length cDNAs of Sp-ALF1 and Sp-ALF2 from mud crab *S. paramamosain*. Tissue distribution determination revealed that Sp-ALF1 and Sp-ALF2 were mainly expressed in mud crab hemoctye. Both the recombinant rSp-ALFs and the synthetic sSp-ALFs peptides exhibited a strong bactericidal activity against Gram-positive and Gram-negative bacteria. The rSp-ALFs also gave antiviral activity against a marine virus-WSSV. At present, increased resistance of bacteria against antibiotic drugs has attracted intensive effort to screen antimicrobial peptides as sources or templates for designing novel therapeutic antibiotics. Regarding its antimicrobial properties, the crab Sp-ALFs appear to be good candidates for further study about their potential use in aquiculture farming and, importantly, as a promising alternative to conventional antibiotics.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.fsi.2012.03.014.

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