Full length article

Characterization of a group D anti-lipopolysaccharide factor (ALF) involved in anti-*Vibrio* response in *Penaeus monodon*

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ABSTRACT

Antimicrobial peptides (AMPs) are an essential component of innate immunity of invertebrates. Anti-lipopolysaccharide factor (ALF), as a main type of AMPs in crustaceans, attends in the disease prevention in general. In this research, a novel Group D ALF was identified and characterized from *Penaeus monodon*, named *Penmon*ALF8. It was an anionic peptide, with both the full-length peptide and lipopolysaccharide binding domain (LBD) a low isoelectric point. *Penmon*ALF8, composed of a signal peptide of 26 amino acids and a mature peptide of 98 amino acids, probably contained three alpha helixes and four beta sheets. Moreover, *Penmon*ALF8 was detected in all tested tissues of *P. monodon*, and the expression level in hemocyte and intestine was relatively high. When challenged by *Vibrio parahaemolyticus*, *Penmon*ALF8 showed 30–100 times higher expression level in all the tissues except in hemocyte and intestine, indicating that *Penmon*ALF8 played a very important role in the immune response of *P. monodon*. By fusing to a SUMO protein, *Penmon*ALF8 was successfully over-expressed in *E. coli* and purified by affinity chromatography. Additionally, the reconstituted *Penmon*ALF8 and its LBD region displayed modest antimicrobial activity. This is the first research about the Group D ALF in *P. monodon*, which provides more information for humoral immunity study of shrimps.

1. Introduction

Antimicrobial peptide (AMP) is a kind of peptide discovered in almost all living organisms [1]. They play very important roles in the innate immunity by killing invading microbes, and regulating other immune or inflammatory response [2]. AMPs exhibit broad-spectrum of antimicrobial activities against variety of bacteria, fungi, parasites, viruses, and even cancer [3]. So far, more than 2960 AMPs have been identified, while 2184 of which are from animals [2].

Invertebrates lack the acquired immunity and rely on the innate immunity to defend against microbes. The innate immunity is constituted by humoral and cellular immunity, while AMP is a main component of the humoral immune system. When the invading microorganisms were detected by host pattern recognition proteins (PRPs), signal transduction pathways were triggered to initiate the production of AMPs [4,5]. In crustaceans, various AMPs were identified, including crustins, penaeidins, anti-lipopolysaccharide factors

(ALFs), stylicins, haemocyanin-derived peptides and lysozymes [6,7].

The first ALF was isolated from the hemolymphs of horseshoe crabs, *Tachypleus tridentatus* and *Limulus polyphemus* [8]. This kind of peptide was named ALF, because it can bind and neutralize the bacterial endotoxin (lipopolysaccharide, LPS) and inhibit the coagulation cascade caused by it [8,9].

ALF normally consists of 120–150 amino acids with a signal peptide and the mature peptide is about 100–130 residues in length. All ALFs contain a conserved disulfide loop formed by two cysteines called lipopolysaccharide binding domain (LBD), as it was supposed to possess LPS-binding activity [10]. The LBD alone can exhibit high antimicrobial activities, *e.g.* the LBDs of ALF2 and ALF7 from *Penaeus chinensis* can inhibit the growth of specific bacteria at 1–2 μM, and LBDs of ALF1, 2, 5, 7 can reduce WSSV copies for about 100 times [11].

In the recent years, many crustacean ALFs have been characterized [12–14]. So far, ALFs have been classified into at least five groups [15,16]. The Group A, B and C ALFs were found in almost all shrimps.

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The seven ALF isoforms from *Penaeus monodon* were classified into Group A (ALF*Pm*1-2), Group B (ALF*Pm*3-5) and Group C (ALF*Pm*6-7) [17]. The Group D ALFs were very anionic (p*I* = 5.58–6.10) peptides, first described in *Litopenaeus stylirostris* [18]. In *Marsupenaeus japonicas*, five groups of ALFs were found, including a new type of ALF called Group E ALF [15]. Different types of ALFs were simultaneously transcribed in one single shrimp, but showed different response trend to microbial challenge [18].

Some Group B ALFs, *e.g. Penmon*ALF3, can effectively inhibit the growth of Gram-positive bacteria, Gram-negative bacteria and fungi at very low concentration [19] and it can also protect the shrimps against white spot syndrome virus (WSSV) infection [20] by binding to the envelope protein WSSV189 [21]. However, the Group D ALF showed impaired LPS binding activities and limited antimicrobial activity compared to Group B ALFs [18].

In this research, a novel Group D ALF from *P. monodon* was discovered and characterized both *in vivo* and *in vitro*. Its response to *Vibrio* challenge was shown and the antimicrobial activity was tested. To our knowledge, this is the first time to study an anionic ALF in *P. monodon*.

2. Materials and methods

2.1. Antimicrobial peptide discovery and bioinformatics analysis

The nucleotide sequence of the new ALF was obtained by transcriptome sequencing of the hepatopancreas of *Penaeus monodon* post challenged with *Vibrio parahaemolyticus* for 3 h (SRA accession: PRJNA473435). The unigene was defined as ALF by blasting with NT, NR, KOG, KEGG and SwissProt databases.

The gene structure of *Penmon*ALF8 was analyzed by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) to obtain the Open Reading Frame and amino acid sequence. SignalP 4.0 server was used to predict the signal peptide. DNAman version 6 was used to show the sequence information. Homology of *Penmon*ALF8 with other ALFs was analyzed by Basic Local Alignment Search Tool (BLASTP). Clustalw (https://www.genome.jp/tools-bin/clustalw) was used for multi-sequences alignment and the results were generated with the online software ESPript 3.0 [22]. Structure of *Penmon*ALF8 was predicted by the Phyre2 web portal for protein modeling, prediction and analysis (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) [23]. The maximum likelihood tree was constructed by MEGA X [24,25] and a bootstrap test was performed [26]. The online software Protparam (http://web.expasy.org/protparam/) was used to predict the physicochemical properties of *Penmon*ALF8.

2.2. Immune challenging of shrimps and tissues collection

50 individual shrimps (about 16 cm in length) were collected from a prawn breeding base (Dapeng, Shenzhen) and divided into two groups. The shrimps were cultured at 25 ± 1 °C in 50 L glass boxes, each containing 10 L of filtered seawater.

Vibrio parahaemolyticus strain OS4 was used to challenge the shrimps. It was a pathogenic strain isolated from a diseased grouper [27] and was kindly provided by Prof. Li Deng. The bacteria were cultured with Luria-Bertani broth containing 3% NaCl shaken at 30 °C to logarithmic phase. After harvested and washed twice with sterilized PBS, 100 μL bacteria (1×10^7 CFU/mL) or PBS were injected into the ventral blood sinus of shrimps in two groups, respectively.

Hepatopancreas of PBS or bacterial challenged shrimps were collected at 0, 2, 4, 8, 12 and 24 h post injection. Stomach, heart, gills, intestine and hemocytes were collected at 4 h post challenge.

The hemocytes were collected by syringing at least 600 μL hemolymph from pericardial sinus of shrimps to 100 μL anticoagulant (0.1 M Sodium Citrate, 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.6) and centrifuging at 830 g, 4 °C for 10 min. After washed once with 1 mL anticoagulant, the collected cells were suspended in 600 μL lysis buffer to

1	TCAGTCAAACACATTAATAATCTACA**ATG**GGTTTGAGTTCAACTTTTGTGAGTGCCGTGT
1	<u>M G L S S T F V S A V</u>
61	TGGTTGTGGCACTGGTGGCACCCTTGACCCCACGATGCTACGGGTTTGGGTTAAAGGACC
12	<u>L V V A L V A P L T P R C Y G</u> F G L K D
121	TCTTTTTACCCGCCATCACGGACCAAGTTAAAGATTTATGGAGAAACGGAGACGTTGATC
32	L F L P A I T D Q V K D L W R N G D V D
181	TGGTCGACCACTCGTGCTCATACAGTGTCAAGCCGGATATACAGGGTATCGAGCTCTATT
52	L V D H S [C S Y S V K P D I Q G I E L Y
241	TCATCGGATCGGTCACGTGCCCGGGCTGGACGACCATCAGGGGAGAGTCCAACACCCGCA
72	F I G S V T C] P G W T T I R G E S N T R
301	GCAAATCCGGGGTTTTGAATGCCGCAATCAAAGACTTTATTCAGAAAGCTCTTAAAGCAG
92	S K S G V L N A A I K D F I Q K A L K A
361	GACTGGTCACCGAAGAGGAAGCAAAACCATATCTCGTA**TAA**TGTGATAGAGATAGGGAGA
112	G L V T E E E A K P Y L V *
421	AT

Fig. 1. Nucleotide and deduced amino acid sequence of *Penmon*ALF8. The initiation and termination codons are bolded; the signal peptide is underlined; the LBD region is marked with a box and the two cysteines are in red.

proceed with the subsequent total RNA extraction.

2.3. Total RNAs extraction and cDNAs synthesis

Total RNAs were extracted using RNeasy Mini Kit (Qiagen, USA), according to the manufacturer's instruction. cDNAs were synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan).

2.4. Confirmation of the penmonALF8 gene

The *penmon*ALF8 gene was amplified by Reverse-transcription PCR (RT-PCR) with primers *Penmon*ALF8 conF (5′ TCAGTCAAACACATTA ATAATCTAC 3′) and *Penmon*ALF8 conR (5′ TCTCCCTATCTCTATCAC ATTATAC 3′). The PCR product was linked to a T-vector (pMD™18-T Vector Cloning Kit, Takara), transformed into *Escherichia coli* TOP 10 strain and sequenced.

2.5. Tissue distribution of PenmonALF8 in P. monodon

Semi-quantitative real-time PCR was performed to test the relative expression level of *Penmon*ALF8 in different tissues of *P. monodon*. One pair of primers (*Penmon*ALF8 F: 5′ATTTATGGAGAAACGGAGACG 3′; *Penmon*ALF8 R:5′ ATTTGCTGCGGGTGTTGGAC 3′) was used and EF-1α (Forward primer:5′ GGACAGCACCGAGCCCAAG 3′; Reverse primer: 5′ TGCTTCTTCCACCAGCCCATA 3′) was used as a control. The cDNAs of hepatopancreas, stomach, heart, gills, intestine and hemocytes from unchallenged shrimps were used as templates. The PCR program was run for initial denaturation at 94 °C for 3 min; followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s, and a final extension at 72 °C for 10 min. The PCR product was then checked by electrophoresis on a 2% agarose gel.

2.6. Response of PenmonALF8 to bacterial challenge

The expression trend of *Penmon*ALF8 in hepatopancreas and other tissues after *Vibrio* challenge was checked by quantitative real-time PCR. The qRT-PCR was performed with SYBR Premix Ex *Taq*II (TaKaRa, Japan) on ViiA7 Real-time PCR system (ABI, USA). The same primers and control gene as in 2.5 were used. The cDNAs from PBS or *V. parahaemolyticus* challenged shrimps were used as templates. The qRT-PCR protocol was as follows: 95 °C, 30 s; 40 cycles of 95 °C, 5 s and 60 °C, 34 s; a melt from 60 °C to 95 °C. The experiment contained three duplicates and gene expression profile was calculated using the
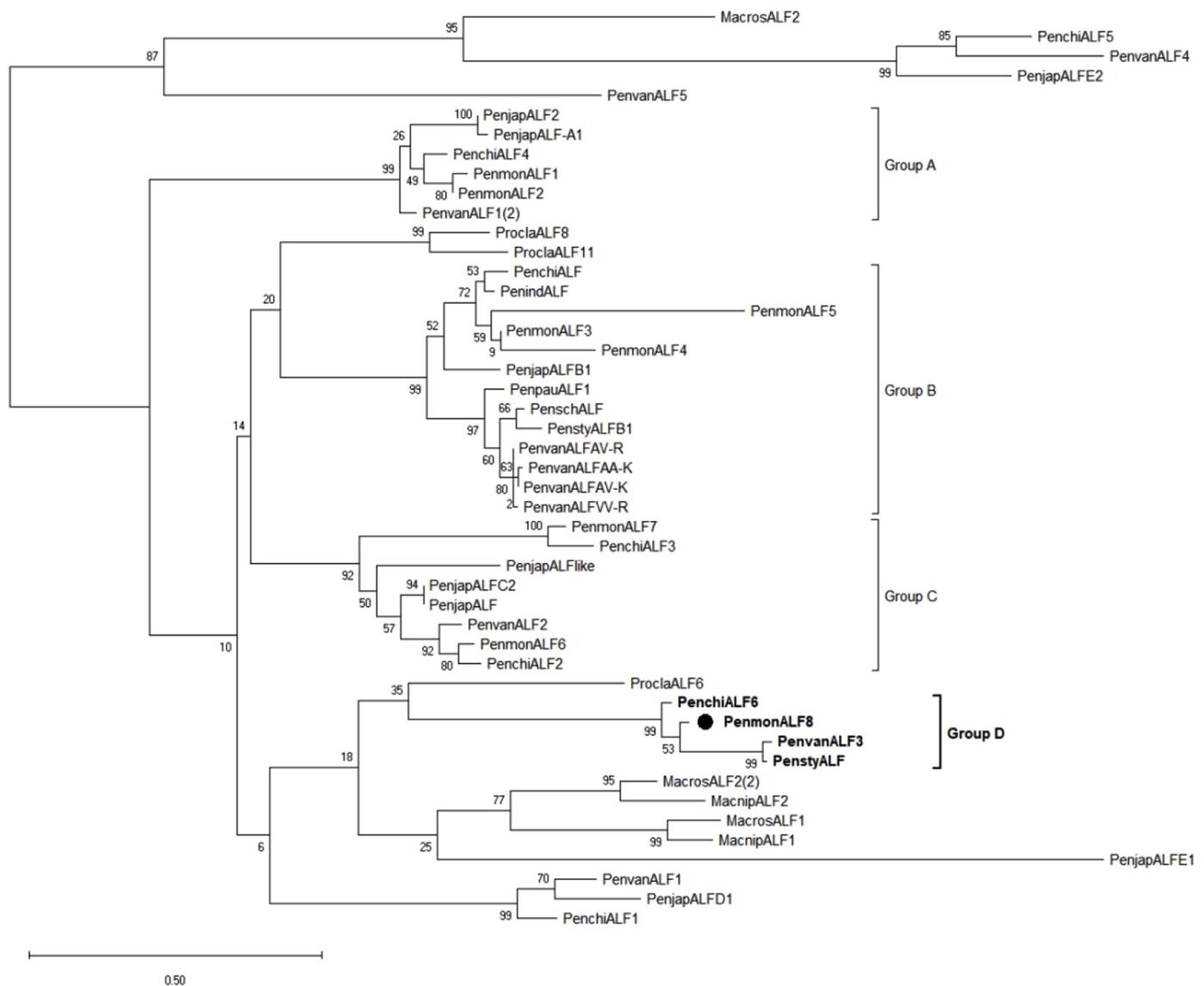
Fig. 2. Molecular Phylogenetic tree of anti-lipopolysaccharide factors from *Penaeus* and *Macrobrachium* analyzed by Maximum Likelihood method conducted in MEGA X. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The bootstrap test was replicated 1000 times. 47 different ALFs were analyzed and classified into four groups. *Penmon*ALF8 was indicated by a black spot. And all Group D ALFs were in bold. The GenBank IDs of ALFs sorted by species are as follows: <u>*Penaeus chinensis*</u> (*Penchi*ALF: AHN13886.1;*Penchi*ALF1: AFU61124.1; *Penchi*ALF2:AFU61125.1; *Penchi*ALF3: AFU61126.1; *Penchi*ALF4: AFU61127.1; *Penchi*ALF5: AFU61128.1; *Penchi*ALF6: AFU61129.1); <u>*Penaeus indicus*</u> (*Penind*ALF: ADE27980.1); <u>*Penaeus japonicas*</u> (*Penjap*ALF2: BAH22585.1; *Penjap*ALF-A1: ANA91278.1; *Penjap*ALFB1: ASR74829.1; *Penjap*ALFC2: AME17862.1; *Penjap*ALFD1: AME17863.1; *Penjap*ALFE1:ASR74830.1; *Penjap*ALFE2: ASR74831.1; *Penjap*ALFlike: BAE92940.1); <u>*Penaeus monodon*</u> (*Penmon*ALF1: ABP73290.1; *Penmon*ALF2: ABP73291.1; *Penmon*ALF3:AEW91477.1; *Penmon*ALF4: ABP73293.1; *Penmon*ALF5: CF415871.1; *Penmon*ALF6: AER45468.1; *Penmon*ALF7: ANP92039.2); <u>*Penaeus paulensis*</u> (*Penpau*ALF1: ABQ96193.1); <u>*Penaeus schmitti*</u> (*Pensch*ALF: ABJ90465.1); <u>*Penaeus stylirostr*</u>is (*Pensty*ALF: AAY33769.1; *Pensty*ALFB1: AGH32549.1); <u>*Penaeus vannamei*</u> (*Penvan*ALF1: AVP74301.1; *Penvan*ALF1': AHG99284.1; *Penvan*ALF2: AVP74302.1; *Penvan*ALF3: AVP74303.1; *Penvan*ALF4: AVP74304.1; *Penvan*ALF5: AVP74305.1; *Penvan*ALFAA-K: ABB22833.1; *Penvan*ALFAV-K: ACT21197.1; *Penvan*ALFAV-R: ABB22832.1; *Penvan*ALFVV-R: ABB22831.1); <u>*Macrobrachium nipponense*</u> (*Macnip*ALF1: ALF02818.1; *Macnip*ALF2: ALF02817.1) <u>*Macrobrachium rosenbergii*</u> (*Macros*ALF1: AFW04304.1; *Macros*ALF2: AOF80304.1; *Macros*ALF2': ADI80707.1).

$2^{-\triangle\triangle CT}$ method with the 0 h PBS treated sample as control for all the other samples.

2.7. Synthesis of the lipopolysaccharide binding domain (LBD) of PenmonALF8

The LBD region of *Penmon*ALF8 with one flanking amino acid residue in each terminal (S**C**SYSVKPDIQGIELYFIGSVT**C**P-NH$_2$) was chemically synthesized by DGpeptides Co. Ltd., (Hangzhou, China). A disulfide bond was formed between the two cysteines and the peptide was amidated in the C terminus. The purity of the peptide was 95%.

2.8. Overexpression and purification of PenmonALF8

The mature peptide part of *Penmon*ALF8 was fused to the C-terminus of a His-SUMO protein (behind the Gly-Gly residue) and overexpressed in *E. coli* BL21 (DE3). The DNA encoding His-SUMO-ALF8 with *Nde*I and *Sac*I cut sites in both ends was chemically synthesized (General Biosystems, Inc., Hefei, China) after codon optimization for *E. coli*. After digested by *Nde*I and *Sac*I, the synthesized DNA was linked to pColdIV vector and the resulting reconstituted plasmid pColdIV-SUMO-ALF8 was transformed into *E. coli* BL21 Rosetta (DE3). The strain was grown at 37 °C until OD600 reached 0.4–0.6, and then induced with 1 mM isopropyl-1-thio-β-D-galactopyranosid (IPTG) at 16 °C for 12 h.

Table 1
pI values and number of charged residues of ALFs from shrimp.

Name	Mature peptide			LBD			Group
	pI	Number of negatively charged residues	Number of positively charged residues	pI	Number of negatively charged residues	Number of positively charged residues	
*Penmon*ALF2	5.46	12	11	9.39	1	4	A
*Penjap*ALF2	5.88	13	11	9.39	1	4	A
*Penchi*ALF4	6.06	13	11	9.39	1	4	A
*Penmon*ALF1	6.07	7	6	9.39	1	4	A
*Penvan*ALF1′	8.62	13	15	9.24	1	4	A
*Penjap*ALF-A1				9.39	1	4	A
*Penpau*ALF1	10.17	8	17	10.04	0	6	B
*Penmon*ALF4	8.18	1	2				B
*Penjap*ALFB1	9.64	9	15	9.70	0	5	B
*Penmon*ALF3	9.95	7	16	9.93	0	6	B
*Penmon*ALF5	9.30	11	17	9.93	0	6	B
*Penind*ALF	10.09	7	16	9.93	0	6	B
*Penvan*ALFAA-K	10.09	9	18	10.04	0	6	B
*Penvan*ALFAV-K	10.09	9	18	10.04	0	6	B
*Penvan*ALFAV-R	10.17	9	18	10.04	0	6	B
*Penvan*ALFVV-R	10.17	9	18	10.04	0	6	B
*Pensch*ALF	10.18	8	17	10.04	0	6	B
*Pensty*ALFB1	10.24	8	18	10.04	0	6	B
*Penchi*ALF	10.29	6	17	9.93	0	6	B
*Penmon*ALF7	8.74	9	11	8.03	0	1	C
*Penjap*ALFC2	9.51	9	13	10.32	0	5	C
*Penmon*ALF6	9.77	8	13	10.32	0	5	C
*Penchi*ALF2	9.18	9	12	10.32	0	5	C
*Penvan*ALF2	9.50	8	12	10.32	0	5	C
*Penchi*ALF3	5.27	11	9	8.68	0	2	C
*Penjap*ALFlike	7.98	12	13	9.84	1	5	C
*Penjap*ALF	9.51	9	13	10.32	0	5	C
*Penvan*ALF3	5.58	13	11	4.03	3	1	D
PenmonALF8	5.26	13	11	4.37	2	1	D
*Penchi*ALF6	5.23	13	11	4.37	2	1	D
*Pensty*ALF	6.10	12	11	4.37	2	1	D
*Macros*ALF2	6.33	14	13	9.14	2	5	other
*Macros*ALF2′	9.10	9	12	9.31	1	4	other
*Procla*ALF11	6.78	10	10	9.31	1	4	other
*Macnip*ALF2	8.68	10	12	9.59	1	5	other
*Macnip*ALF1	8.00	13	14	9.70	1	5	other
*Macros*ALF1	5.08	15	14	9.84	1	5	other
*Procla*ALF6	7.94	12	13	8.76	2	4	other
*Procla*ALF8	7.99	10	11	9.31	1	4	other
*Penjap*ALFE2	5.33	15	12	9.31	2	5	other
*Penchi*ALF5	5.64	14	12	9.24	2	5	other
*Penjap*ALFE1	9.27	12	17	5.82	1	1	other
*Penvan*ALF1	7.09	11	11	9.39	1	4	other
*Penjap*ALFD1	9.2	13	17	9.24	1	4	other
*Penchi*ALF1	8.64	11	13	8.82	1	3	other

Afterwards, cells were harvested by centrifugation at 8000*g* for 10 min. The collected cells were suspended with 30–50 mL balance buffer (50 mM Tris-HCl, 200 mM NaCl, pH 8.0) and disrupted by sonication (40 min, 200 W, 2 s on, 4 s off). The cell lysate was centrifuged at 10000 g, 4 °C for 35 min to separate the supernatant and sediment. The fusion protein was purified by NGC Chromatography System (Bio-rad, USA) equipped with a Ni-NTA Sepharose Fast Flow (Ruidahenghui, Beijing, China) column. After the supernatant was loaded, the column was washed with 60 mM imidazole in balance buffer, and the target protein was eluted with 200 mM and 500 mM imidazole in balance buffer. The eluted protein was further dialyzed against the balance buffer to remove the imidazole and then quantified by Bradford reagent (Sangon, China). SDS-PAGE on a precast 4–20% gel (GenScript Biotech Corp., Nanjing, China) was performed to check the expression and purification result.

2.9. Removal of SUMO tag and intact protein mass measurement by LC-ESI-MS

To remove the SUMO tag, 100 μg of the target protein was incubated with 1U SUMO protease (General Biosystems, Inc., Hefei, China) at 4 °C overnight. The digested product was checked by SDS-PAGE on a precast 4–20% gel (GenScript Biotech Corp., Nanjing, China) and LC-ESI-MS. The protein sample was first desalted with Zeba™ Spin Desalting Columns, 7K MWCO (Thermo Scientific, USA) and then applied to a C4 RP-HPLC column and eluted with a linear gradient of 20–65% acetonitrile, containing 0.1% formic acid in 15 min at a flow rate of 300 μL/min. TOF-MS scan was used to obtain MS data on Triple TOF 5600 plus mass spectrometer (Sciex, Concord, Ontario, Canada) equipped with electrospray ion source. Some mass spectrometer acquisition parameters were set as followings: ion spray voltage of 5.5 kV, nebulizer gas of 55 psi, auxiliary gas of 45 psi, an interface heater temperature of 150 °C, declustering potential of 120 V, scan mass range from *m/z* 600 to *m/z* 3000, accumulation time of 1 s, and time bins to sum of 80. Acquired mass spectra were automatic reconstructed by Peakview software (v2.2) to calculate the intact protein mass.

2.10. Antimicrobial activity of PenmonALF8 and LBD of PenmonALF8

The indicator strains were cultured until OD600 reached 0.4.
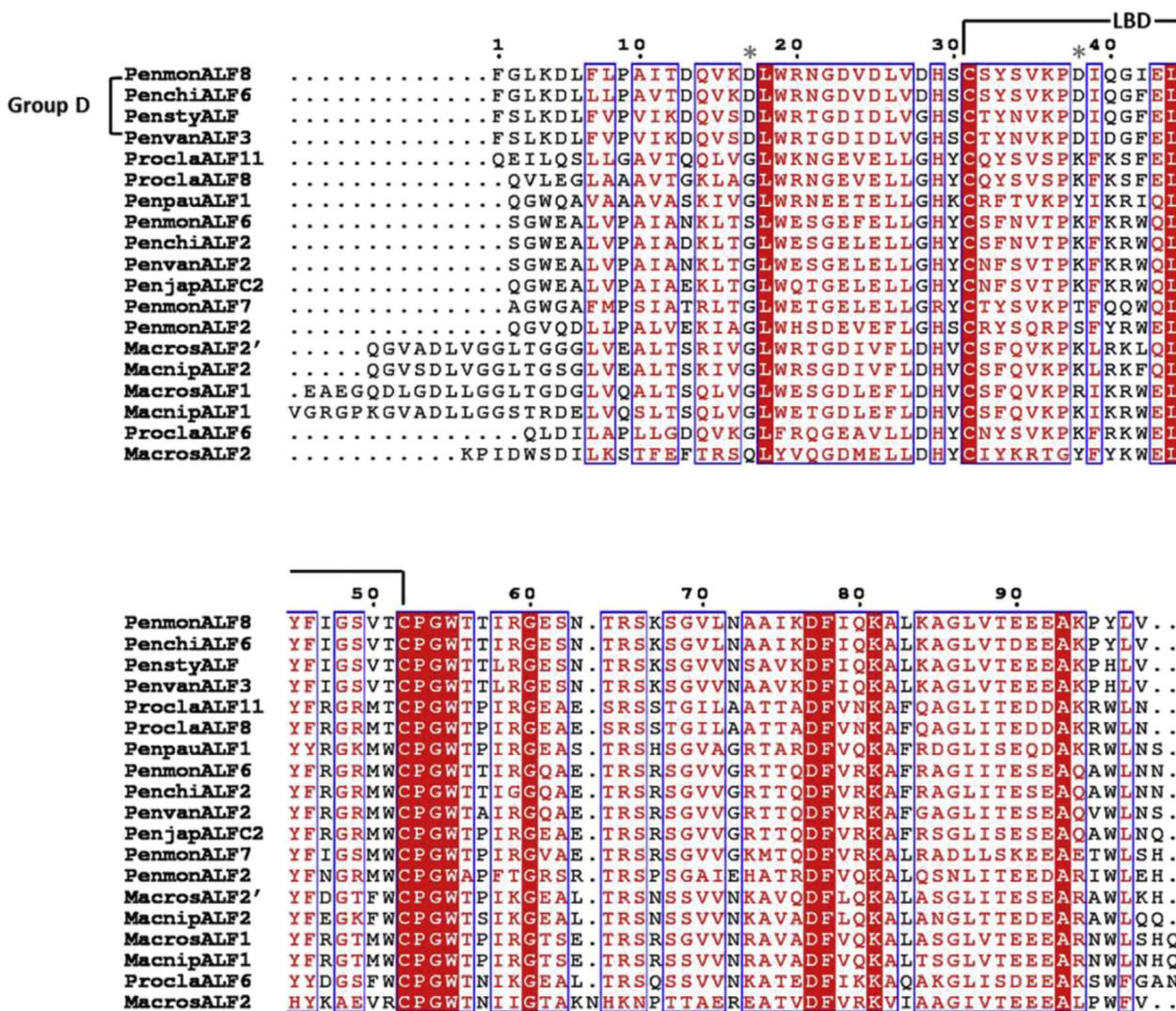
Fig. 3. Alignment of *Penmon*ALF8 with other ALFs. Identical residues were in red background; similar residues were in red. The two aspartic acids were marked with asterisks. Group D ALFs and LBD region were indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
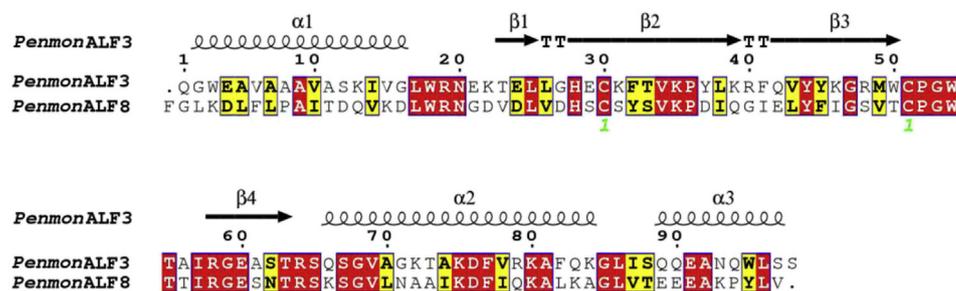
Fig. 4. Alignment of *Penmon*ALF8 and *Penmon*ALF3 (PDB ID: 2JOB). Secondary structure of *Penmon*ALF3 was shown. α, α-helix; β, β-sheet; T, hydrogen bonded turn. The two cysteines were labeled with a green "*1*". (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Bacillus sp., *Exiguobacterium* sp. and *Pantoea* sp. were collected by our laboratory from lake and cultured with LB medium shaken at 37 °C. *Mesorhizobium* sp. was isolated from sea and cultured with 2216E medium (Tryptone 5 g, yeast extract 1 g, FePO$_4$ 0.1 g, filtered sea water 1 L, pH 7.6) shaken at 37 °C. *E. coli* JM107 and *Staphylococcus aureus* were cultured with LB medium shaken at 37 °C. The cultured strains (For the activity of full-length *Penmon*ALF8, only *Exiguobacterium* sp., *S. aureus* and *Mesorhizobium* sp. were used as indicator strains) were diluted 1000 times with relevant medium and then mixed with synthesized LBD of *Penmon*ALF8 or purified full-length *Penmon*ALF8 in a 96-

well plate. The final concentration of the peptide was 64 μM. PBS and 500 μg/mL ampicilin were used as negative and positive control, respectively. The plate was incubated at 37 °C for 18 h and OD600 as checked at 0 h and 18 h. The experiment was repeated three times.

2.11. Statistical analysis

GraphPad Prism 5 was used to perform the statistical analysis of expression profiles and generate the tables. Student's t-test was used to calculate the statistical difference between two treatments. $p < 0.05$
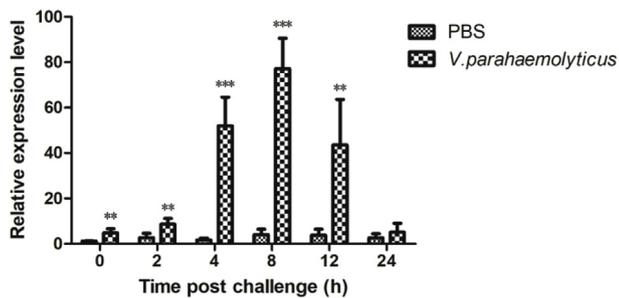
Fig. 5. Expression patterns of *Penmon*ALF8 in hepatopancreas of *P. monodon* after *V. parahaemolyticus* challenge. Relative expression level was analyzed by qRT-PCR. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The asterisks highlight the significance of difference analyzed by Student's t-test (**, $P < 0.01$; ***, $P < 0.001$).
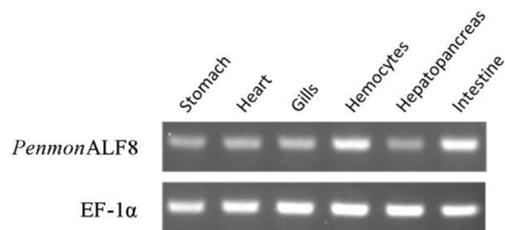
Fig. 6. Tissue distribution of *Penmon*ALF8 in *P. monodon* tested by semi-quantified RT-PCR. EF-1α was used as an internal reference.
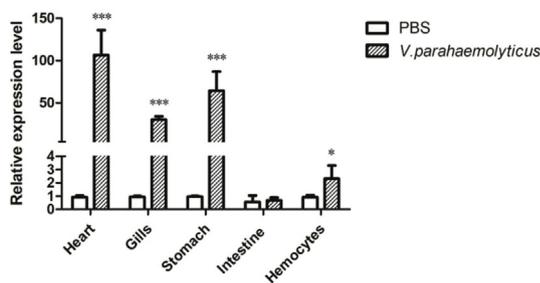
Fig. 7. Relative expression level of *Penmon*ALF8 in five different tissues post challenge. Tissues were taken from shrimps injected with *V. parahaemolyticus* or PBS for 4 h qPCR was used to test the relative expression level. The asterisks highlight the significance of difference analyzed by Student's t-test (*, $P < 0.05$; ***, $P < 0.001$).

was considered as significant different.

3. Results

3.1. Identification of a new ALF from P.monodon

The new type of anti-lipopolysaccharide factor was discovered by blasting the trancriptome of hepatopancreas of *P. monodon* (SRA accession: PRJNA473435) with NT, NR, KOG, KEGG and SwissProt database. As there were in total 7 ALFs reported in *P. monodon*, this new ALF was named *Penmon*ALF8. The nucleotide sequence of *Penmon*ALF8 was confirmed by RT-PCR. The full-length gene of *Penmon*ALF8 was 375 bp, encoding a 124-residues peptide which contained a 26 residues signal peptide and a mature peptide. The LPS-binding domain (LBD) with two conserved cysteines was from residue 57 to 78 (Fig. 1). The molecular weight of the mature peptide was 10.8 kDa.

3.2. PenmonALF8 is classified into group D ALF

Full length *Penmon*ALF8 showed 94% identity to ALF6 from *Penaeus*

chinensis, 85% and 84% identity to ALF from *Penaeus stylirostris* and ALF3 from *Penaeus vannamei*, respectively. However, it had much lower identity to other ALFs (lower than 60%). The phylogenetic tree of ALFs from shrimps (Fig. 2) showed that these four ALFs were on a new branch distinguished from other ALFs and could be clustered to Group D ALF. As described by Rafael Diego Rosa et al., ALFs of Group D are very anionic polypeptide (calculated pI = 5.58–6.10). The mature peptide of these four ALFs all had low pI (Table 1) and the pI value of their LBD was even lower, which indicated that they were typical anionic ALFs. The mature peptide of some Group A ALF showed low pI value (5.46–6.07), but their LBD region were very cationic (p$I > 9$). Both the mature peptide and LBD of Group B ALF were highly cationic (pI around 10). And except *Penchi*ALF3, all calculated Group C ALFs were positively charged in both the mature peptide and LBD region.

3.3. Sequence similarity and structure analysis of PenmonALF8

Sequence alignment of *Penmon*ALF8 with 18 other ALFs from shrimps (Fig. 3) showed that the conservatism of shrimp ALFs was relatively high, with 12 of 98 residues very conserved in all ALFs, 60 residues identical and the rest 26 residues diverse. Notably, the N terminal part and the LBD region of Group D ALFs were unique to other ALFs, especially the D17 and D38 residues, which make the peptide more anionic.

Structure prediction of *Penmon*ALF8 conducted by Phyre2 software showed that it could have a similar 3D structure to *Penmon*ALF3 (PDB ID: 2JOB). The sequence identity of them was 41% and they had 100% confidence to be homogenous. It probably contained one α-helix structure in the N-terminus, several β-sheets in the middle and two α-helixes in the C-terminus. Alignment of these two ALFs and secondary structure of *Penmon*ALF3 are shown in Fig. 4.

3.4. Response of PenmonALF8 to Vibrio challenge

As shown in Fig. 5, the expression level of *Penmon*ALF8 in hepatopancreas of *P. monodon* significantly increased when the shrimp was challenged by *V. parahaemolyticus*. Significant upregulation was detected at 4–12 h post challenge. And then the expression returned to almost the normal level before 24 h. This is in accordance with the transcriptome sequencing result, which showed that the expression of *Penmon*ALF8 was up-regulated 12 times at 3 h post challenge.

3.5. Tissue distribution of PenmonALF8 in P. monodon

Tissue distribution of *Penmon*ALF8 in *P. monodon* was tested by semi-quantified RT −PCR. Result (Fig. 6) showed that *Penmon*ALF8 was distributed in all tissues tested, but the expression level in hemocytes and intestine was relatively higher.

Expression level of *Penmon*ALF8 in different tissues changed when the shrimp was challenged by *V. parahaemolyticus* for 4 h (Fig. 7). Compared to the control, 30–110 times higher expression level was detected in heart, gills and stomach of challenged shrimps. However, the expression level was equal in intestine, and in hemocytes it was only about 3 times higher than the control. As the normal expression level of *Penmon*ALF8 in intestine and hemocytes was relatively high, the results indicate that *Penmon*ALF8 was highly expressed in all tested tissues post *Vibrio* challenge.

3.6. Recombination expression of PenmonALF8

*Penmon*ALF8 was successfully expressed and purified in *E. coli*, by fusing with a His-tagged SUMO protein [28] and using the pColdIV vector (Fig. 8). Most of the recombinant protein was expressed in the supernatant when the host was induced with IPTG at low temperature (Fig. 8A). The fusion protein was further purified by a Ni-NTA column to about 95% purity (Fig. 8B). The SUMO tag was further cut off by a
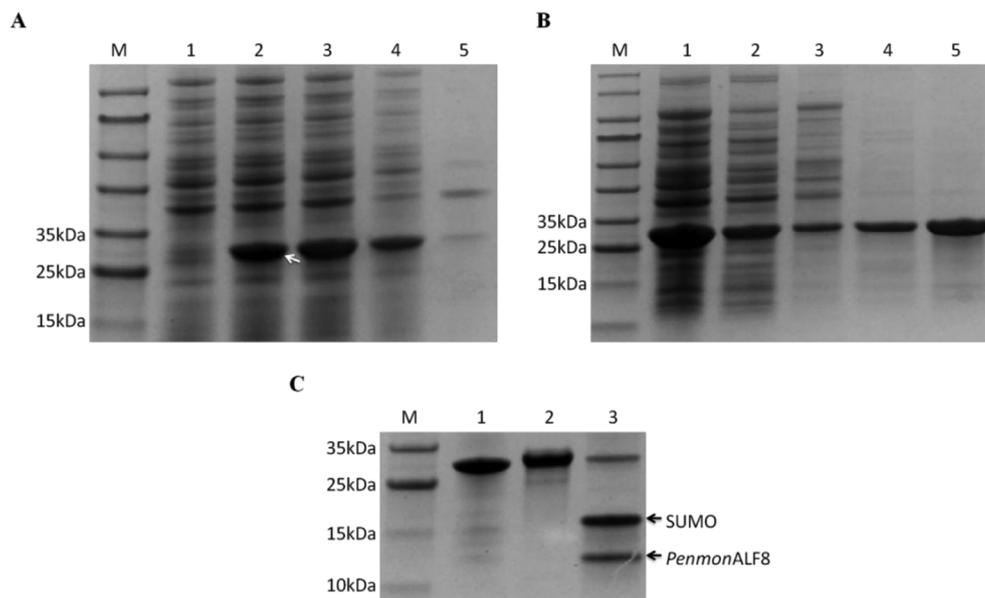
Fig. 8. Recombination expression and purification of *Penmon*ALF8 analyzed by SDS-PAGE. A, Over-expression of His-SUMO-*Penmon*ALF8 in *E. coli* (BL21). M, Protein marker; 1, total protein of *E. coli* without induction; 2–3, total protein of *E. coli* induced with 1 mM IPTG at 16 °C for 6 and 12 h, the fusion protein is indicated by an arrow; 4, supernatant after cell disruption; 5, sediment after cell disruption. B, Purification of *Penmon*ALF8 by Ni-NTA column. M, Protein marker; 1, sample, which is the supernatant after cell disruption; 2, flowthrough; 3, wash off protein with 60 mM imidazole; 4–5, eluted proteins with 200 mM and 500 mM imidazole. C, Cleavage of the SUMO tag. M, Protein marker; 1, purified protein before cleavage; 2, SUMO protease; 3, protein after cleavage, with SUMO and *Penmon*ALF8 indicated.
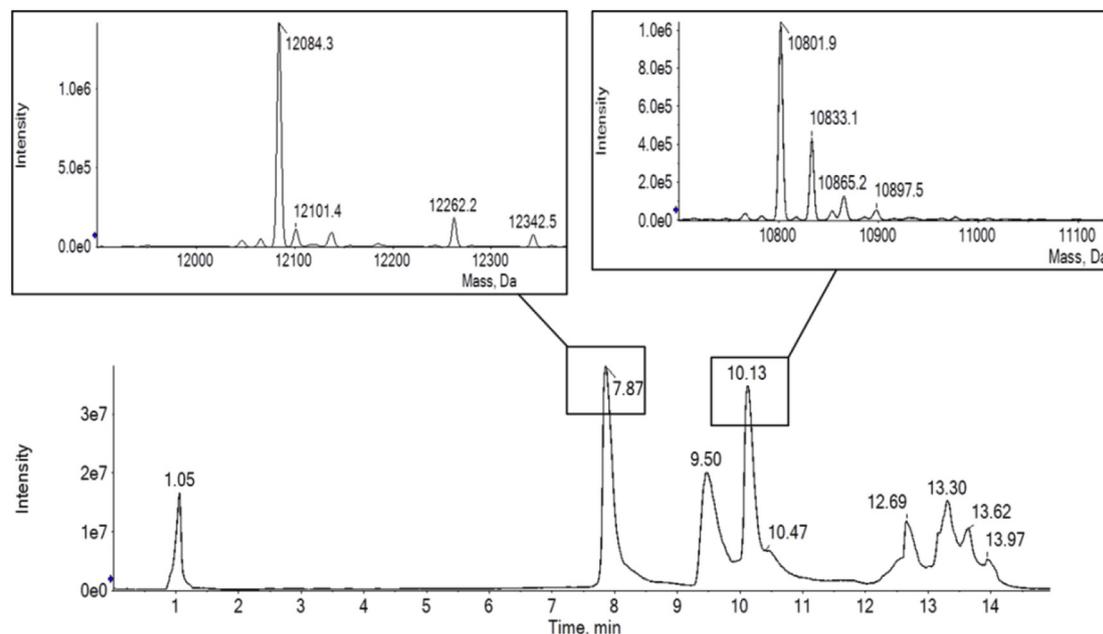
Fig. 9. LC-ESI-MS analysis of the enzyme digested product of SUMO-*Penmon*ALF8. The UV-absorbance curve of HPLC and the MS results of 7.87 min and 10.13 min peaks were shown.

SUMO protease (Fig. 8C). SDS-PAGE showed that molecular weight (MW) of the fusion protein, SUMO and *Penmon*ALF8 was relatively larger than the theoretical MW, so LC-MS was performed to confirm the MW of the molecules. The result (Fig. 9) showed that there were two main constituents in the digested product, with a MW of 12084.3 Da and 10801.9 Da, which was in accordance with the theoretical MW of SUMO (12084.53 Da) and *Penmon*ALF8 containing one disulfide bond (10801.34 Da), respectively. This indicated that the SUMO tag was precisely cut off and *Penmon*ALF8 was expressed correctly with one disulfide bond formed.

3.7. Antimicrobial activity

The bactericidal activity of the LBD region and full-length *Penmon*ALF8 was compromised and they could only slightly inhibit a few types of Gram-positive and Gram-negative bacteria at 64 μM

(Fig. 10), while all the tested bacteria except *Mesorhizobium* sp. C1R were sensitive to ampicillin. The LBD region of *Penmon*ALF8 showed the most significant inhibitory activity against a type of *Bacillus* isolated from lake. The full-length *Penmon*ALF8 displayed higher inhibition activity than the LBD region against *Exiguobacterium* sp. L33 and *Mesorhizobium* sp. C1R.

4. Discussion

In this study, a new anti-lipopolysaccharide factor was discovered in *P. monodon*. This ALF was different from the seven ALFs described in *P. monodon* previously, as it was an anionic peptide. It showed a conserved sequence and structure character of ALF. A main difference was that this ALF had more positively charged residues than negatively charged residues in both the LBD region and full-length peptide. In the classification of ALFs, *Penmon*ALF8 was clustered into Group D ALF. So far,
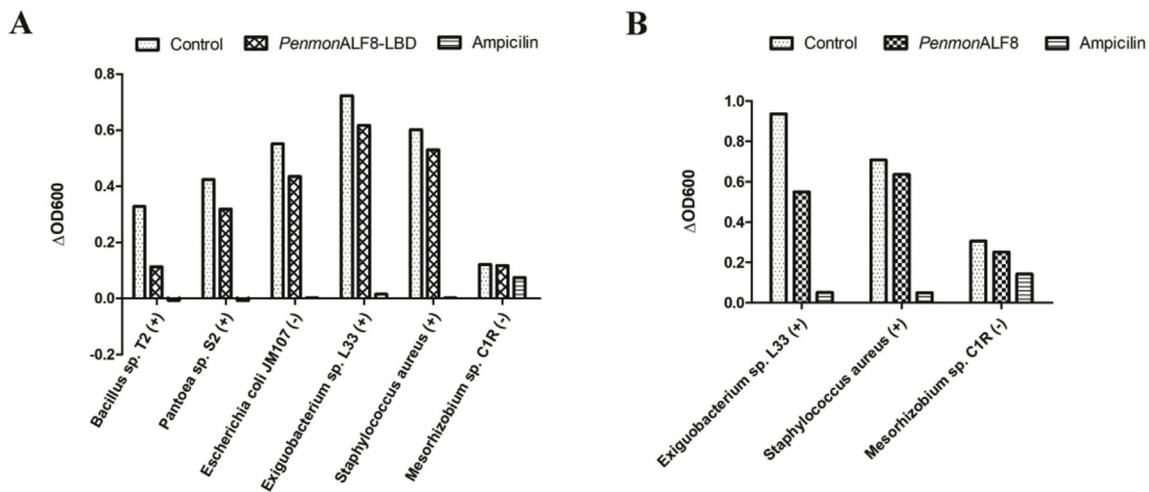
Fig. 10. Antimicrobial activity of *Penmon*ALF8-LBD (A) and full-length *Penmon*ALF8 (B) indicated by the change of turbidity of indicator strains. The Gram-positive and negative type of the strains was indicated with a (+) or (−). The experiment was repeated thrice and same results were obtained.

there are four Group D ALFs discovered from four different types of shrimps. All ALFs in this group had low p*I* value in both the LBD region and full-length peptide. Some ALFs, *e.g.* Group A ALFs only showed low p*I* in full-length peptide. And this can be a crucial reference to classify ALFs in future studies. The *Penjap*ALFD1 was grouped to Group D ALF previously [15], however, it was positively charged actually. So in this research, it was clustered to other group. In a recent study, an ALF with the same sequence as *Penmon*ALF8 was described and also named ALF*Pm*8 [29], but not experimentally characterized.

There were normally several types of ALFs simultaneously expressed in a single shrimp, while most of them were cationic ALFs. As the only negatively charged ALF in one shrimp, the function of Group D ALF is of great interest. In this research, we found that *Penmon*ALF8 was expressed normally in a relatively high level in intestine and hemocytes, while in other tissues the expression level of *Penmon*ALF8 post *Vibrio* challenge was much higher than the normal expression level. This indicated that *Penmon*ALF8 could be an essential component of humoral immunity during anti-*Vibrio* response in *P. monodon*. And it played an important role in all tissues. As a control, PBS was injected and it did not affect the expression of *Penmon*ALF8, which was checked in the pre-experiment (data not shown). A previous study of Julien de Lorgeril et al. also showed that the relative expression of ALF-D1 in circulating hemocytes of *P. stylirostris* during *Vibrio penaeicida* infection was up-regulated (about 2–3 fold) at 12 h and 24 h post infection [30]. It was reported previously that *Litvan* ALF-D did not show an apparent increase of expression in hemocytes post challenged by a fungal pathogen *Fusarium solani* [18], which means that Group D ALF might not response to fungi challenge, but do participate in the anti- *Vibrio* immune reaction.

ALFs possess efficient anti-bacterial activity, and yeast was normally used as an expression host [19,31], *e.g. Penmon*ALF3 and *Penchi*ALF2 were successfully expressed in *P. pastoris*. However, it takes long time. SpALF6 of mud crab *Scylla paramamosain* was expressed solubly in *E. coli* with pET-30a expression vector [12]. But in most cases, ALFs form inclusion bodies when expressed in *E. coli*, *e.g. Litsty*ALF-D1 [18], *Penjap*ALFE2 [15], *Penchi*ALF5 [13]. In this research, we use protein fusion method to express ALF. By fusing to a SUMO protein, *Penmon*ALF8 was expressed as a soluble protein. Using the pColdIV vector and performing the induction at low temperature could be an optimal way to express ALFs, as we had tried to express *Penmon*ALF8 with pSmart-SUMO vector at 37 °C, but the fusion protein was not soluble (data not shown).

The antimicrobial activity of *Penmon*ALF8 was relatively low compared to other ALFs [13,31,32]. Similar results were obtained with

*Litsty*ALF-D1, which can only inhibit the growth of *Bacillus megaterium* (IBMC collection) and *Escherichia coli* SBS363 and its LBD region did not show any antimicrobial activity [18]. On the contrary, some positively charged ALFs exhibit efficient bactericidal activity. *Penmon*ALF3 can bind to LPS and lipoteichoic acid (LTA) with high affinity [33], and it can induce bacterial membrane permeabilization, disruption, damage, bleb, pore formation and leakage of cytoplasmic components [32]. It has been shown that basic residues are important for the binding and antimicrobial activity of ALF, and increasing the number of lysine in the LBD region can significantly enhance the inhibition activity [14,34,35]. Therefore, the impaired activity of *Penmon*ALF8 is probably due to a lack of positively charged residues. To improve the antimicrobial activity of *Penmon*ALF8, basic residues could be added to substitute some non-polar residues. Furthermore, the solubility of synthesized LBD was low (data not shown), which was detrimental for the bactericidal activity.

In summary, although *Penmon*ALF8 did not show an effective antimicrobial activity, it might play other roles during *Vibrio* infection. Further studies could be conducted to reveal the functions of Group D ALFs in the innate immunity of shrimps.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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