Identification and characterization of two highly homologous lysozymes from red swamp crayfish, *Procambarus clarkii*

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ABSTRACT

Lysozyme is an important immune effector in innate immunity against pathogen infection. But the study on the active region of lysozyme is limited. In this study, two highly homologous lysozymes were identified from crayfish (designated as PcLysi4 and PcLysi5). The molecular structures of PcLysi4 and PcLysi5 were predicted by SWISS-MODEL with the structure of lysozyme (PDB accession No. 4PJ2.2.B) as model. The results suggested that the structure of PcLysi4 and PcLysi5 were highly similar, but there were more α-helices at positions (127–139) and longer β-sheet at positions (49–57) in the structure of PcLysi5 than in that of PcLysi4. The antibacterial and antiviral functions of the two lysozymes were investigated. PcLysi4 and PcLysi5 could promote the bacterial clearance ability of crayfish, and increase the survival rate of *Vibrio*-infected crayfish. Further study showed that PcLysi5 inhibited WSSV replication, and enhanced the survival rate of WSSV-infected crayfish. There was no evidence that PcLysi4 has an influence on WSSV replication. Furthermore, PcLysi5 was detected to interact with envelope protein VP24 of WSSV. Our results would provide a new reference for the study on active region of lysozyme.

1. Introduction

Although lacking adaptive immunity, invertebrates are mostly depended on innate immunity to resist pathogenic infection. The innate immune system of crayfish include cellular and humoral responses [1]. Antimicrobial peptides (AMPs) play important roles in innate immunity of crayfish. Lysozyme, a glycolytic enzyme, is well known as an AMPs with antibacterial activity against both gram-negative and gram-positive bacteria [2-5].

Lysozyme is an important immune effector in antibacterial immune response. The antibacterial activity of lysozyme is attributed to its hydrolyze activity to the β−1, 4-glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan [5]. Lysozyme hydrolyzes bacterial cell wall peptidoglycan, leading to bacterial cell lysis [6]. Increasing number of studies have reported that

lysozyme could also act as accessory factors to exert antimicrobial effects. Such as, lysostaphin re-sensitizes multidrug-resistant *Staphylococcus aureus* to β-lactams both *in vitro* and *in vivo* [7]. Previous study has revealed that lysozyme could interact with antibiotic, and enhance the effect of antibiotic [8]. Furthermore, PcLys-i3, a lysozyme derived from *Procambarus clarkii*, was reported to bind to ferritin to exert bacterial killing [9].

Much of the literature has reported that lysozyme participate in antiviral immune response. Mj-ilys, a lysozyme identified from *Marsupenaeus japonicus*, was upregulated after white spot syndrome virus (WSSV) challenge [10]. LYZ1 inhibits infection of WSSV by interacting with several viral structural proteins in *Litopenaeus vannamei* [11]. A c-type lysozyme, derived from *Fenneropenaeus penicillatus*, possessed antiviral activity against WSSV and IHHNV [12]. Previous study suggested that lysozyme derived from chicken egg white, human milk and

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human neutrophils possess activity against HIV-l [13]. Moreover, lysozyme was also reported to act as anticancer agent [14].Number of studies have reported that lysozyme interacts with anticancer drugs pinostrobin, methotrexate, 6-mercaptopurine and ellipticine to enhance the anticancer activity of these drugs [15-18].

Lysozyme participate in multiple immune responses, but active region of lysozyme in immune response needs to be further studied. In this study, two novel lysozymes were identified from *P. clarkii* (designated as PcLysi4 and PcLysi5). PcLysi4 and PcLysi5 are highly homologous. We compared the structure and analysis the roles of them in the immune response to investigate the active region of lysozyme. These results provide a new reference for antimicrobial mechanism of AMPs.

2. Materials and methods

2.1. Bioinformatics analyses

Expressed sequence tags (ESTs) of PcLysi4 and PcLysi5 were obtained through transcriptome sequencing of crayfish. Multiple alignment was conducted by using MEGA 6.0. Molecular modeling of PcLysi4 and PcLysi5 were performed using SWISS MODEL with the structure of lysozyme (PDB accession No. 4PJ2.2.B) as the model.

2.2. Tissue distribution and expression profiles

The qRT-PCR was conducted to detect the mRNA tissue distribution and expression profiles of PcLysi4 and PcLysi5 with the according primers. The 18S rRNA was used as the reference control with the
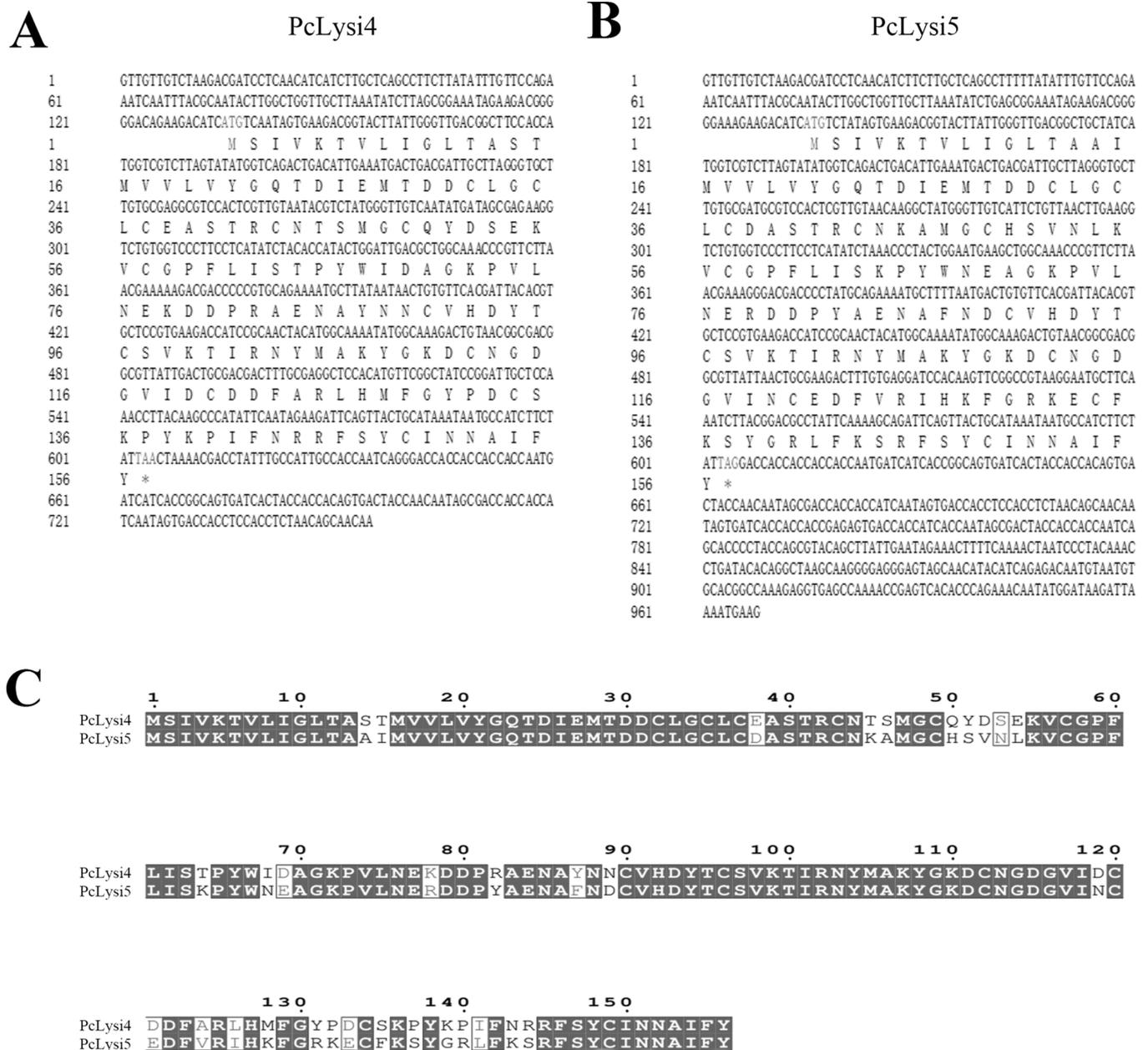
Fig. 1. Nucleotide sequences and amino acid sequences of PcLysi4 and PcLysi5
(A) Nucleotide sequences and amino acid sequences of PcLysi4. PcLysi4 has 755 bp, with a 471 bp open reading frame encoding for 156 AA. (B) Nucleotide sequences and amino acid sequences of PcLysi5. PcLysi5 has 968 bp, with a 471 bp open reading frame encoding for 156 AA. (C) Alignment of PcLysi4 and PcLysi5. The alignment was conducted by using MEGA 6.0.

A
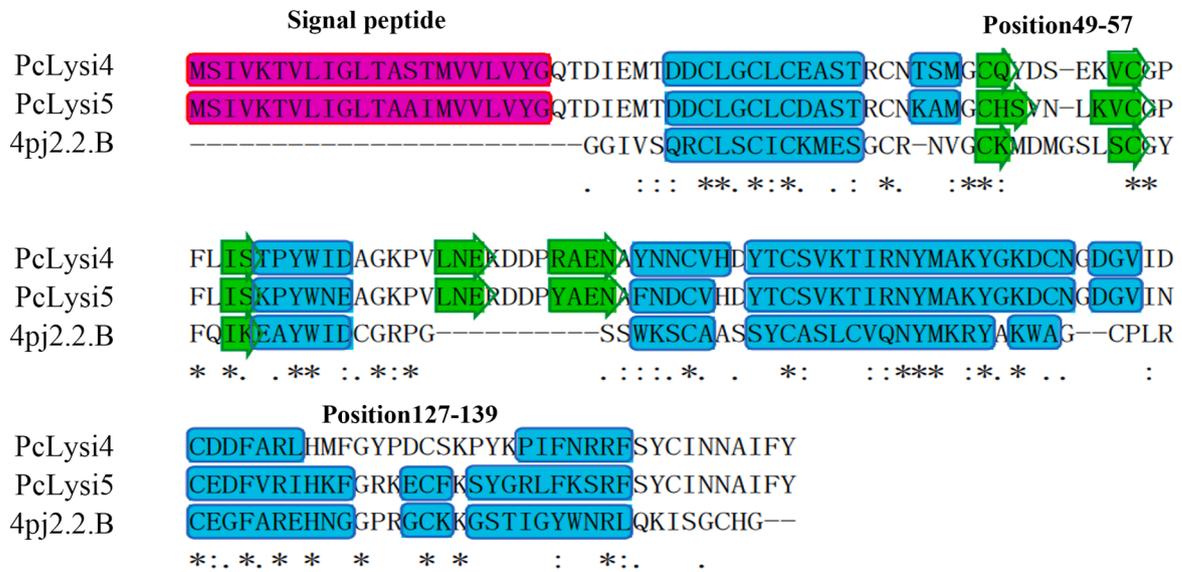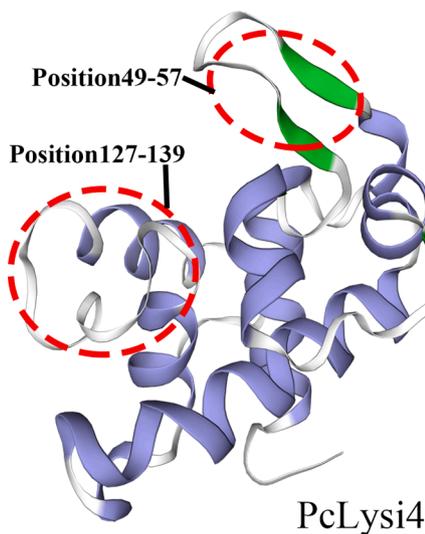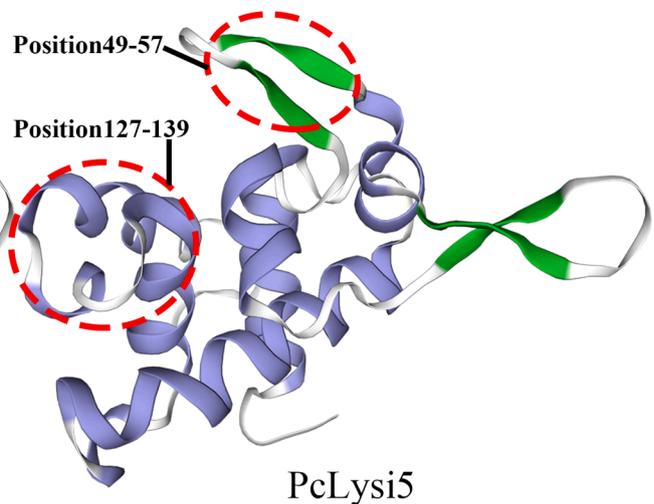
Fig. 2. Structural model of PcLysi4 and PcLysi5
(A) Multiple alignment of the sequences of PcLysi4, PcLysi5 and the model lysozyme (PDB accession No. 4pj2.2.B). (B) and (C) are respectively the structural model of PcLysi4 and PcLysi5. Molecular modeling were performed using SWISS-MODEL with the structure of lysozyme (PDB accession No. 4pj2.2.B) as model. The α-helixes are in blue, β-sheets are in green, and signal peptides are in red.

primers 18S F/R. In the mRNA tissue distribution test, the total RNA of heart, hepatopancreas, gills, stomach, intestine and muscle from three normal crayfish, were extracted using TRIzol reagent (CWBio, China). The cDNA was synthesized with a reverse transcription kit (Vazyme, China) according to the manufacturer's instruction. In the expression profiles assays, the crayfish were respectively challenged with PBS, WSSV, *Vibrio parahemolyticus* and *Staphylococcus aureus*. Then, total RNA was extracted, and the cDNA was synthesized. The cDNA was used as template for the qRT-PCR detection. Threshold cycle method was used to calculate the results.

2.3. Recombinant protein expression and purification

PcLysi4 and PcLysi5 were amplified with the according primers. The products and the vector (pET-32a and pGEX-4T-1) were digested with the corresponding restriction enzymes (Thermo, USA) at 37 °C for 30 min and ligated into the vectors using T4 DNA ligase (Thermo, USA). The recombinant plasmid of VP24 is obtained from our previous study [19]. The recombinant plasmid transformed into *E. coli* DH5α (Trans-Gen, China) firstly for confirming the inserted nucleotide sequence by DNA sequencing, and then into *E. coli* BL21 (DE3) (TransGen, China). The recombinant PcLysi4, PcLysi5 and VP24 were purified with His-Bind resin or GST-Bind resin (Novagen, Germany) according to previous method [20].

2.4. Bacterial clearance assay

Crayfish were injected with His-Tag, His-PcLysi4 or His-PcLysi5 protein. One hour after injected with protein, twenty μl 1×10^9 CFU *V. parahaemolyticus* were injected. Thirty minutes after bacteria
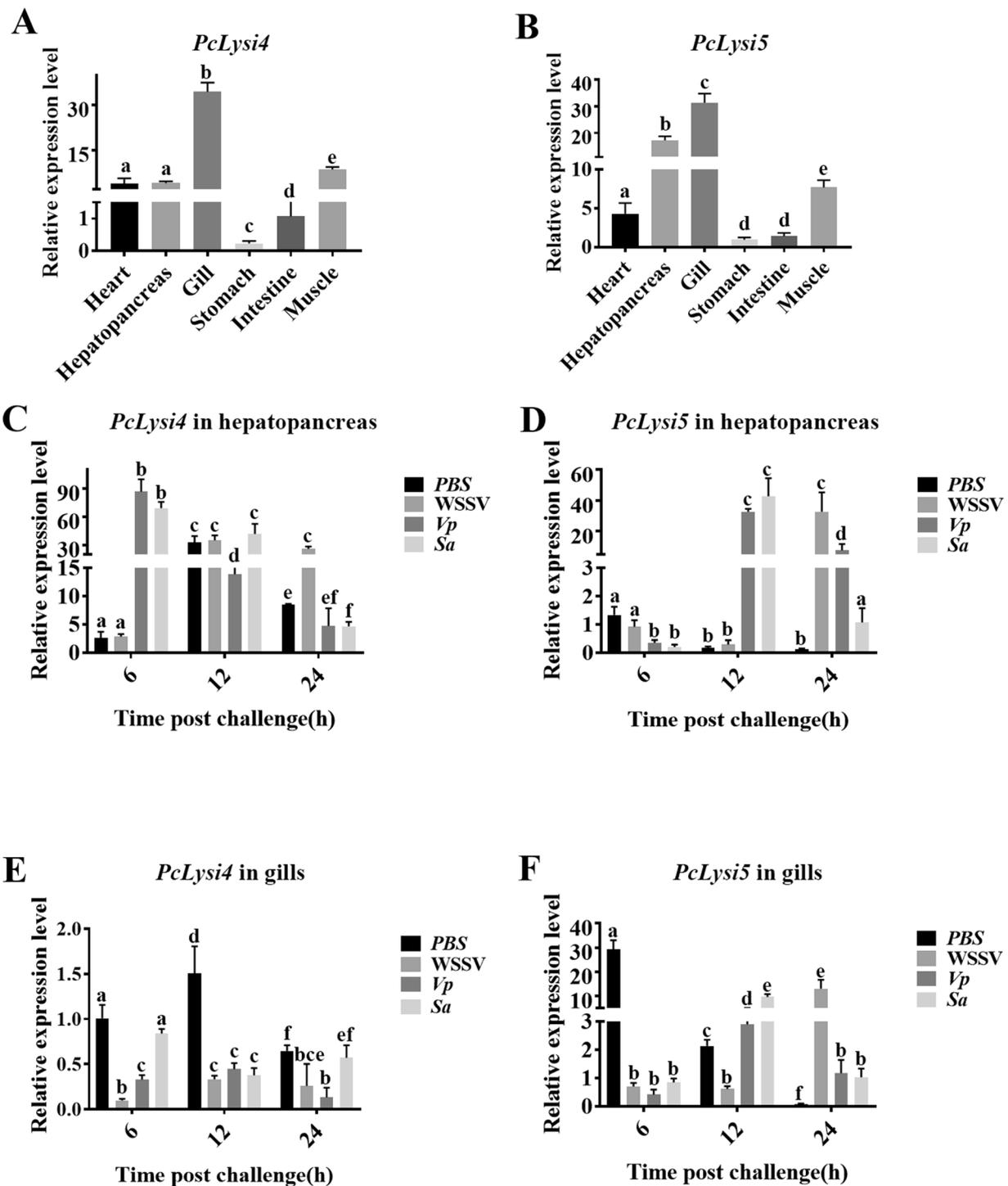
Fig. 3. The mRNA tissue distribution and expression profiles of PcLysi4 and PcLysi5
The qRT-PCR was used, and 18S RNA was used as internal control in these assays. (A) The mRNA tissue distribution of PcLysi4. (B) The mRNA tissue distribution of PcLysi5. (C) The expression profiles of PcLysi4 in hepatopancreas after challenged with PSB, WSSV, *S. aureus* and *V. parahemolyticus*. (D) The expression profiles of PcLysi5 in hepatopancreas after challenged with PSB, WSSV, *S. aureus* and *V. parahemolyticus*. (E) The expression profiles of PcLysi4 in gills after challenged with PBS, WSSV, *S. aureus* and *V. parahemolyticus*. (F) The mRNA expression profiles of PcLysi5 in gills after challenged with PBS, WSSV, *S. aureus* and *V. parahemolyticus*. Differences between groups were analyzed using one-way analysis of variance (ANOVA). Different letters indicate significant differences ($p < 0.05$).

injection, hemolymph of crayfish was collected and diluted in PBS, then cultured on solid LB plates overnight. The numbers of bacterial colonies were counted and the bacterial concentrations in hemolymph were calculated.

2.5. Bacterial binding assay

The recombinant protein of PcLysi4 and PcLysi5 were also used in bacterial binding assay. Three gram-positive bacteria (*S. aureus, S. agalactiae* and *B. subtilis*), and three gram-negative bacteria (*V. parahaemolyticus, A. hydrophila* and *E. piscicida*) were used to perform bacterial binding assay. His-Tag, His-PcLysi4 or His-PcLysi5 protein
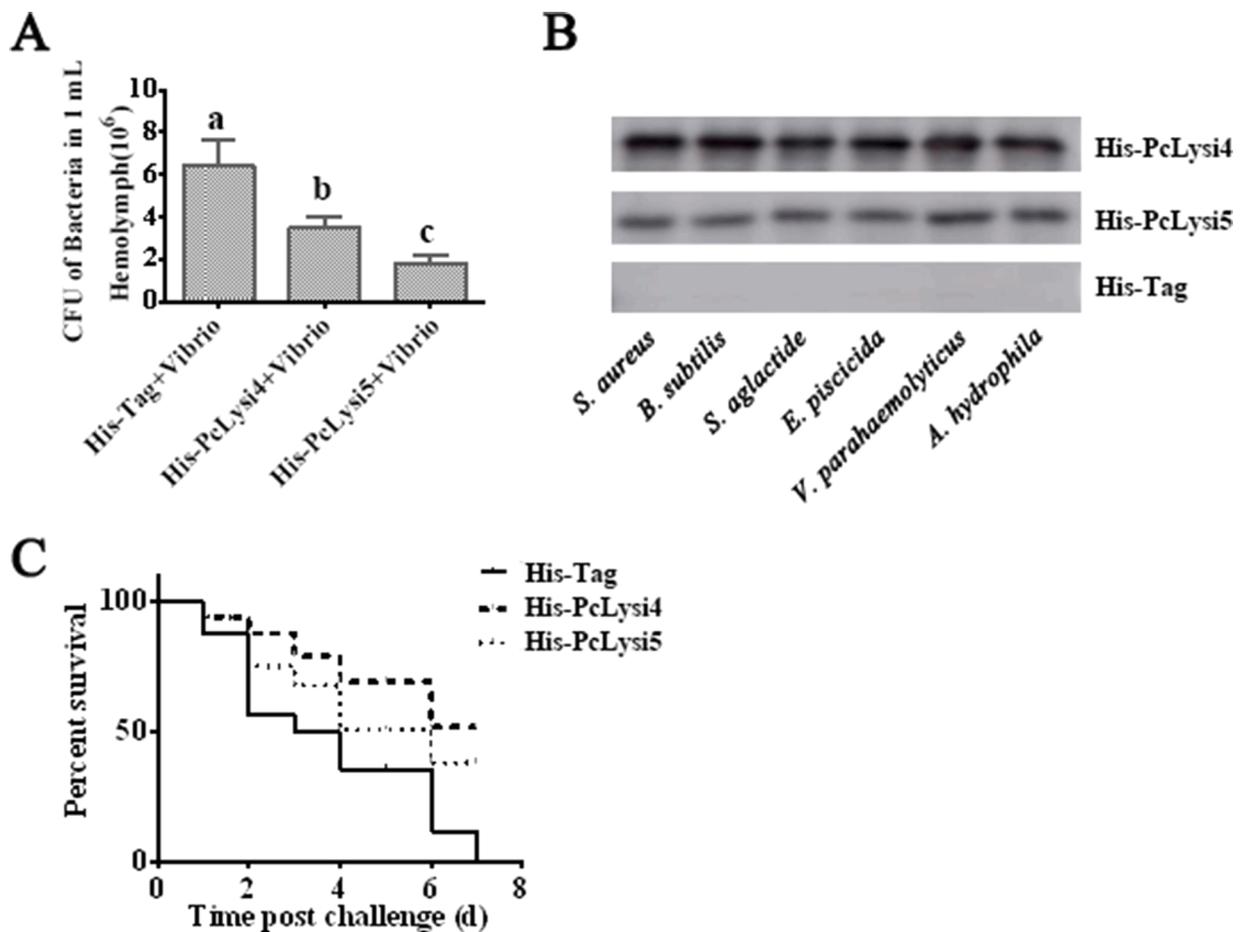
Fig. 4. Both of PcLysi4 and PcLysi5 possessed antibacterial activity *in vivo*
(A) PcLysi4 and PcLysi5 both enhanced the bacterial clearance of crayfish. The hemolymph was collected and diluted in PBS 30 min after bacteria injection, then cultured on solid LB plates overnight. (B) PcLysi4 and PcLysi5 bound to three gram-positive bacteria and three gram-negative bacteria. His-PcLysi4, His-PcLysi5 and His-Tag were respectively incubated with bacteria, and detected with anti-His-antibody after extensively washed. (C) PcLysi4 and PcLysi5 enhanced the survival rate of *Vibrio*-infected crayfish. The crayfish were keep at 25 °C in survival assay. Differences between groups were analyzed using one-way analysis of variance (ANOVA). Different letters indicate significant differences (*p*< 0.05).

were incubated with 5×10^7 CFU resuspended bacteria at 37 °C for 30 min at a final concentration of 50 µg/ml. Then, the mixtures were washed five times with PBS by centrifugation at 5000 g for 5 min resuspended in 50 µl of PBS. Then, mixed with SDS-PAGE sample buffer and boiled for 5 min. The samples were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane for immunoblot analysis. Anti-His-antibody (Frdbio, China) was used in immunoblot analysis.

2.6. Survival assay

The tested crayfish were injected with 50 µl His-Tag, His-PcLysi4 or His-PcLysi5 protein (1 mg/ml), and then injected with *V. parahaemolyticus* (3×10^7 CFU/ml) or WSSV 1 h after the injection of protein. Thirty crayfish were used in every group. The number of dead crayfish was counted every day. All the crayfish were kept at approximately 25 °C.

2.7. Antibacterial activity assays

Gram-positive bacteria (*B. subtilis* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *V. parahaemolyticus*) were used to test the potential antibacterial activity of recombinant His-PcLysi4 and His-PcLysi5. Briefly, 20 µl (5×10^8 CFU/ml) of bacteria was added into 96 well plate and 20 µl of His-Tag, His-PcLysi4 or His-PcLysi5 protein was added, followed by 160 µl of LB medium. After cultured at 28 °C for 10 h,

the absorbance values at OD 600 of bacterial culture medium were detected by enzyme standard instrument.

2.8. Western blot analysis

Western blot was used to detect replication of WSSV after injected with His-Tag, His-PcLysi4 or His-PcLysi5. Tissue proteins were obtained from gill, mixed with SDS-PAGE sample buffer and boiled for 5 min. Then, the samples were resolved by 12% SDS-PAGE and transferred onto nitrocellulose membrane for immunoblot analysis. The membranes were blocked for 1 h with 3% non-fat milk in TBS (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) and incubated with 1/100 diluted antiserum against VP24, VP26, VP28 or β-Actin for 4 h. The antiserums of VP24, VP26, VP28 and β-Actin were prepared in our lab. HRP-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS) was added after washing to remove the free, nonspecifically binding antiserum. Then the membrane was detected with eECL western blot kit (Cwbio, China). The corresponding antiserum were derived from our previous study [19].

2.9. Pull down assay

The pulldown assay was conducted according previous method [20]. The experiment was carried out as follows. The purified GST-PcLysi4 or GST-PcLysi5 were incubated with settled His-binding resin (GenScript, China) in a 1.5 ml EP tube for 1 h at 4 °C and washed with PBS for three
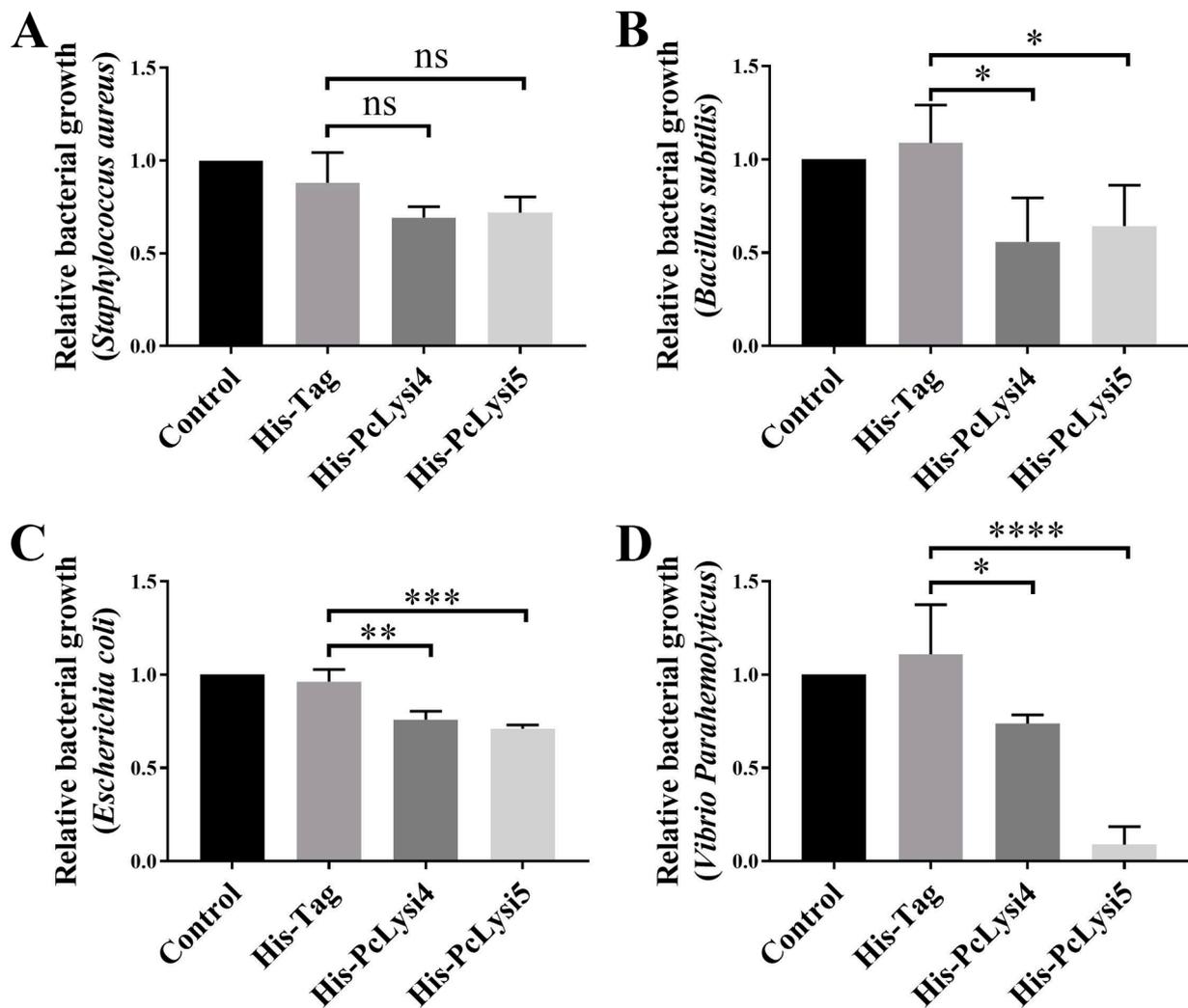
Fig. 5. PcLysi4 and PcLysi5 showed antibacterial activity *in vitro*

The influence of recombinant PcLysi4 and PcLysi5 to bacterial growth were detected by using enzyme standard instrument. Two gram-positive bacteria and two gram-negative bacteria were used in this assay. *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.

times. Then His-VP24 was added into the tube and incubated at 4 °C for 2 h. Finally, the reaction mixture was washed with PBS three times, mixed with SDS-PAGE sample buffer, boiled for 5 min and analyzed with 12% SDS-PAGE.

3. Results

3.1. Bioinformatics analyses

PcLysi4 has 755 bp, with a 471 bp open reading frame encoding for 156 AA (Fig. 1A), and PcLysi5 has 968 bp, with a 471 bp open reading frame encoding for 156 AA (Fig. 1B). PcLysi4 and PcLysi5 showed high similarity (Fig. 1C). Lysozyme (PDB accession No.4PJ2.2.B) was used as model to predict the structures of PcLysi4 and PcLysi5. Multiple alignment of PcLysi4, PcLysi5 and the model lysozyme were performed (Fig. 2A). The structural models of PcLysi4 and PcLysi5 were respectively shown in Fig. 2B and Fig. 2C. Signal peptides are marked in red, α-helixes are marked in blue and β-sheets are marked in green. These results suggested that there were more α-helixes at the position (127–139) and longer β-sheets at the position (49–57) in the structure of PcLysi5 than that in PcLysi4 (Fig. 2B, 2C). The structural differences between PcLysi4 and PcLysi5 promoted us to investigate whether there were differences in immune function between PcLysi4 and PcLysi5.

3.2. Tissue distribution

To investigate the immune function of PcLysi4 and PcLysi5, we detected the tissue distribution of them by qRT-PCR. PcLysi4 and PcLysi5 were detected in all tested tissues. PcLysi4 were highly expressed in gills and muscle, and PcLysi5 were highly expressed in gills and hepatopancreas (Fig. 3A, 3B). Notably, the mRNA expression levels of PcLysi4 and PcLysi5 were both highest in respiratory organs gills, and lowest in digestive tract stomach and intestine.

3.3. Expression profiles of PcLysi4 and PcLysi5

To evaluate the functions of PcLysi4 and PcLysi5, the mRNA expression profiles of PcLysi4 and PcLysi5 in gills and hepatopancreas were detected by qRT-PCR. Further statistical tests revealed that PcLysi4 and PcLysi5 were upregulated after the stimulation of crayfish with WSSV, *S. aureus* or *V. parahaemolyticus* (Fig. 3C, 3D, 3E, 3F), indicating that PcLysi4 and PcLysi5 might participate in the antibacterial response or antiviral response in crayfish. In hepatopancreas, the mRNA expression levels of PcLysi4 were unregulated at 6 h time points after challenged with *Vp* or *Sa*, and that of PcLysi5 were unregulated at 24 h time points after challenged with *Vp, Sa* or WSSV. In gills, the mRNA expression levels of PcLysi5 were upregulated at 24 h time points after challenged with *Vp, Sa* or WSSV.
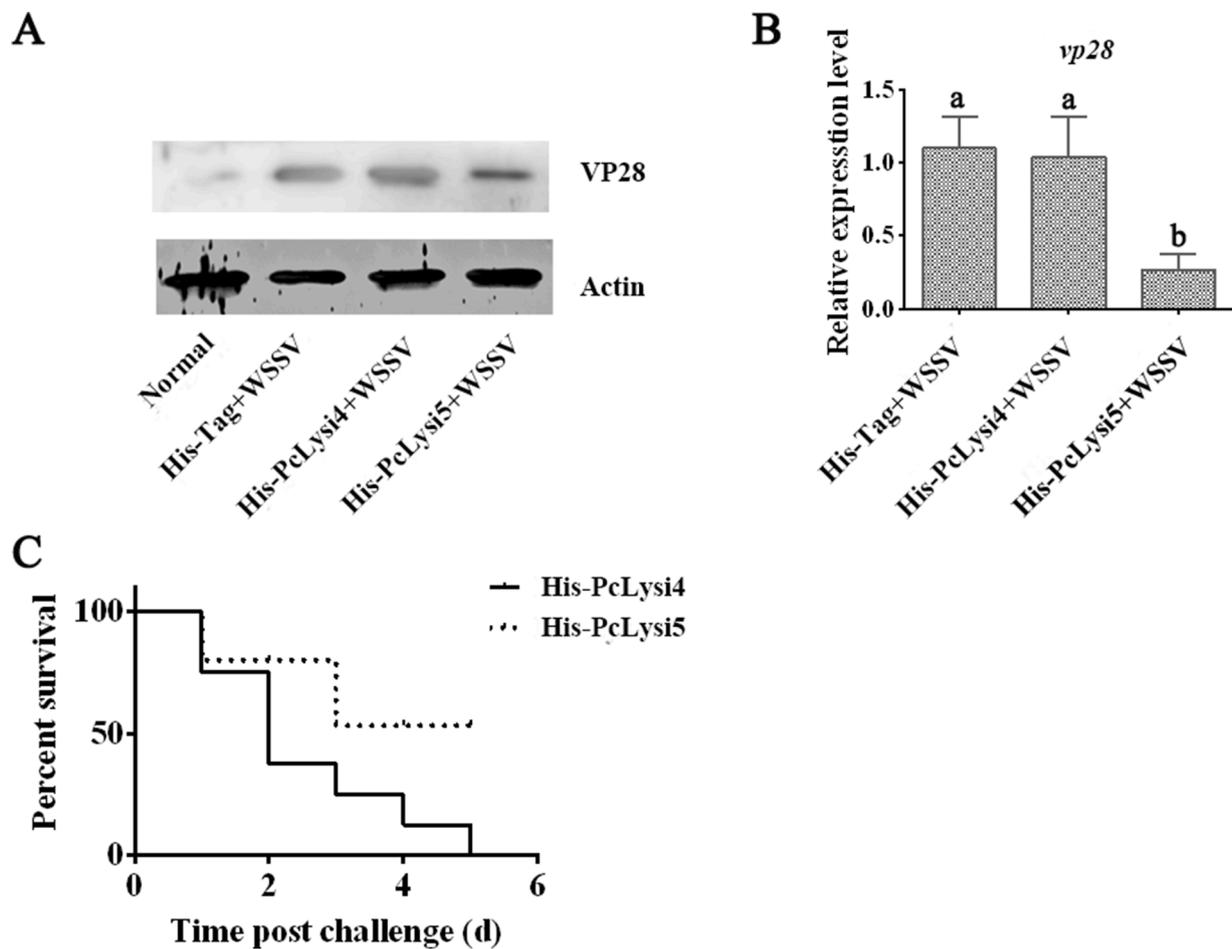
Fig. 6. PcLysi5 possessed antiviral activity *in vivo*

(A) Western blot was used to detect the amount of WSSV, and envelope protein VP28 was used as a marker. (B) The qRT-PCR was used to detected the amount of WSSV, and envelope protein *vp28* gene was used a marker. (C) His-PcLysi5 enhanced the survival rate of WSSV-infected crayfish. The crayfish were keep at 25 °C in survival assay. Differences between groups were analyzed using one-way analysis of variance (ANOVA). Different letters indicate significant differences ($p < 0.05$).

3.4. Antibacterial activity of PcLysi4 and PcLysi5 in vivo

In order to verify whether PcLysi4 or PcLysi5 process antibacterial activity *in vivo*, bacterial clearance assay, bacterial binding assay and survival assay were conducted in this study. In bacterial clearance assay, both of PcLysi4 and PcLysi5 enhance the bacterial clearance ability of crayfish, and more obvious effect was observed in PcLysi5-injected group than in PcLysi4-injected group (Fig. 4A). The bacterial concentrations in His-Tag injected group, PcLysi4-injected group and PcLysi5-injected group were respectively about 6.2×10^6 CFU/ml, 3.8×10^6 CFU/ml and 2.1×10^6 CFU/ml. In the bacterial binding assay, PcLysi4 and PcLysi5 bound to three gram-positive bacteria (*S. aureus, S. agalactiae and B. subtilis*) and three gram-negative bacteria (*V. parahaemolyticus, A. hydrophila and E. piscicida*) (Fig. 4B). Recombinant proteins PcLysi4 and PcLysi5 both improved the survival rate of *Vibrio*-infected crayfish (Fig. 4C). The survival rate in control group was 0, but the survival rate in PcLysi4-injected group and PcLysi5-injected group were respectively about 52% and 45% 7 d post *Vibrio* infection. These results suggested that PcLysi4 and PcLysi5 might participate in antibacterial immune responses of crayfish.

3.5. Antibacterial activity of PcLysi4 and PcLysi5 in vitro

To observe antibacterial activity of PcLysi4 and PcLysi5 *in vitro*, bacteriostatic experiments were conducted. PcLysi4 and PcLysi5 both possess antibacterial activity against gram-positive bacteria (*B. subtilis*) and two gram-negative bacteria (*V. parahaemolyticus and E. coli*) (Fig. 5). PcLysi4 and PcLysi5 did not possess significant antibacterial activity against *S. aureus*. Notably, the PcLysi5 was observed to have strong antibacterial activity against *V. parahaemolyticus*.

3.6. Antivirus activity of PcLysi4 and PcLysi5

The mRNA expression levels of PcLysi4 and PcLysi5 were up regulated after the stimulation of crayfish with WSSV (Fig. 3), so the antiviral functions of PcLysi4 and PcLysi5 were investigated in this study. The viral amounts were analyzed via qRT-PCR and western blot respectively using *vp28* gene and VP28 protein as marker. As shown in Fig. 6A, 6B, PcLysi5 inhibited the replication of WSSV, since the amounts of VP28 and *vp28* gene in PcLysi5-injected group were lower than that in control group. No obvious effect was observed in PcLysi4-injected group.

3.7. PcLysi5 binds to VP24

Increasing number of studies suggested that antibacterial peptides interacted with viral proteins to exert antiviral function [11, 21]. So, pull-down assays were conducted to investigate the interaction between PcLysi4 or PcLysi5 and viral proteins. Recombinant protein PcLysi4 or PcLysi5 were used to pull down natural viral proteins. The results suggested that recombinant protein PcLysi5 interacted with envelope protein VP24, but could not bind to VP26 and VP28. Recombinant protein PcLysi4 could not bind to VP24, VP26 and VP28 (Fig. 7A, 7B, 7C, 7D).
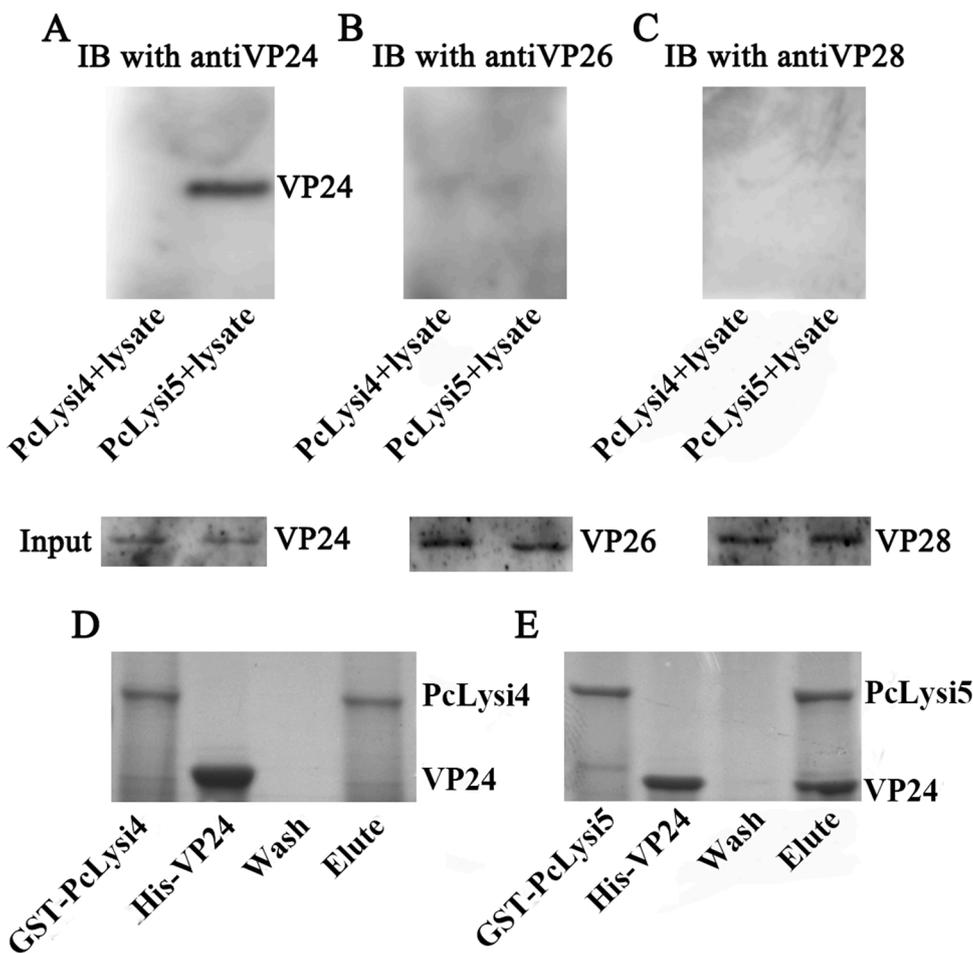
Fig. 7. PcLysi5 interacted with WSSV protein VP24

(A) Pull -down of VP24 using His-PcLysi4 or His-PcLysi5. (B) Pull-down of VP26 using His-PcLysi4 or His-PcLysi5. (C) Pull-down of VP28 using His-PcLysi4 or His-PcLysi5. His-PcLysi4 or His-PcLysi5 were incubated with His-Bind resin, then incubated gill lysate, mixed with SDS-PAGE sample buffer and boiled for western blot analysis. The samples were respectively detected with anti-VP24, anti-VP26 and anti-VP28 serum. (D) Pull-down of His-VP24 using GST-PcLysi4. (E) Pull-down of His-VP24 using GST-PcLysi5.

Furthermore, recombinant protein VP24 was used to verify the interaction between PcLysi5 and VP24 by using pull down assay. Fig. 7D and 7E are respectively shown the pull down of His-VP24 using recombinant GST- PcLysi4 or GST- PcLysi5.

4. Discussion

Lysozyme, an important kind of AMPs, is found in both prokaryotes and eukaryotes[1].AMPs usually have common features, such as a small size, either a linear or cyclic structure. The linear structure encompasses amphipathic α-helices, while the cyclic structure contains one or more disulfide bonds forming a β-sheet [22]. Both of α-helices and β-sheets were found in both PcLysi4 and PcLysi5 in protein structure prediction (Fig. 2). PcLysi4 and PcLysi5 are highly homologous, but there have certain differences between PcLysi4 and PcLysi5 in structure as shown in Fig. 2. Notably, PcLysi5 contains more α-helices and β-sheets structure.

Table 1
Sequences of the primers used in this study.

Primers	Sequences (5′−3′)
EX-PcLysi4-F	TACTCAGAATTCATGTCAATAGTGAAG
EX-PcLysi4-R	TACTCACTCGAGATAGAAGATGGCATT
EX-PcLysi5-F	TACTCAGAATTCATGTCTATAGTGAAG
EX-PcLysi5-R	TACTCACTCGAGATAGAAGATGGCATT
RT-PcLysi4-F	GGTTGTCAATATGATAGCGA
RT-PcLysi4-R	CTTCTATTGAATATGGGCTT
RT-PcLysi5-F	GGTTGTCATTCTGTTAACTT
RT-PcLysi5-R	GATTTGAAGCATTCCTTACG
RT-18S-F	TCTTCTTAGAGGGATTAGCGG
RT-18S-R	AAGGGGATTGAACGGGTTA

Then, we detected the functions of PcLysi4 and PcLysi5 to investigate the influence of structural differences to immune activities. The lysozyme usually exerts antibacterial activity through the hydrolysis of the glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan [23]. Four kinds of lysozyme have been identified from *P. clarkii*, one of which (PcLyzc) shared high similarity to the other known invertebrate c-type lysozymes [9, 24, 25]. Pclysi2 doesn't have antibacterial activity, and other three kinds of lysozyme Pclysi1, Pclysi3, and PcLyzc have antibacterial activity in *P. clarkii*. Whereas, in this study, PcLysi4 and PcLysi5 were upregulated after bacterial stimulation, and both possessed antibacterial activity *in vivo* and *vitro* (Fig. 3, 4, 5), and PcLysi5 shown stronger antibacterial activity to *Vibrio* than PcLysi4.

AMPs were also reported to participate in antiviral immune response in shrimp. FcALF2, one isoform of ALF from *F. chinensis*, possess significant inhibition activity against WSSV [26]. In our study, PcLysi5 was detected to inhibit the replication of WSSV, but no obvious effect was observed in PcLysi4-injected group in crayfish. Several studies have revealed that AMPs interact with viral proteins to inhibit viral replication. FcALF5, an anti-lipopolysaccharide derived from *F. chinensis*, was reported to interact with the WSSV envelope protein VP24 to inhibit WSSV replication [21]. LYZ1, an lysozyme of L. *vannamei*, binds to WSSV structure proteins (VP26, VP28, wsv134 and wsv321) to inhibit viral replication [11]. Our results revealed that PcLysi5 interacted with VP24 to inhibit WSSV replication, but could no interact with VP26 and VP28 (Fig. 7). Though PcLysi4 and PcLysi5 are highly homologous, PcLysi4 could not bind to VP24, VP26 or VP28 (Fig. 7). Previous study revealed that VP24 was involved in the early stage of WSSV infection, and interacted with VP28 which is the major envelope protein of WSSV [27]. The VP24-truncated isolate of white spot syndrome virus possessed

lower infectivity in viral infection [28]. Furthermore, several previous studies suggested that VP24 interacted with several pathogen-associated molecular pattern during WSSV infection [29-31]. Herein, we discovered two highly homologous lysozyme from crayfish. There was a difference between the two isoforms of lysozyme in the affinity to VP24 (Fig. 7). That difference could be the results of the structural difference between the two isoforms of lysozyme (Fig. 2).

The α-helixes are important for protein-protein interaction. The proline is unique as its secondary amine forms a tertiary amide when incorporated into biopolymers, thus preventing hydrogen bond and α-helixes formation [32]. There were two prolines in the amino acid sequence of PcLysi4 at positions (132, 137). That might affect the affinity of PcLysi4 to viral proteins. These findings have significant implications for the understanding of active region of lysozyme (Table 1).

Declaration of Competing Interest

None

Acknowledgements

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