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A new antimicrobial peptide isoform, *Pc*-crustin 4 involved in antibacterial innate immune response in fresh water crayfish, *Procambarus clarkii*

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ABSTRACT

The main advantage of antimicrobial peptides (AMPs) used as the effectors in the innate immunity system of invertebrates is that the high specificity is not indispensable. And they play important roles in the systemic defenses against microbial invasion. In this study, a new full-length cDNA of the crustins molecule was identified in red swamp crayfish, *P. clarkii* (named *Pc-crustin 4*). The ORF of *Pc-crustin 4* contained 369 bp which encoded a protein of 122 amino acids, with a 20-amino-acid signal peptide sequence. On the base of the classification method established by Smith et al., *Pc*-crustin 4 belonged to Type I crustin molecule. The *Pc-crustin 4* transcripts were expressed in hemocytes at relatively high level, and relatively low level in hepatopancreas, gills, and intestine in normal crayfish. After respectively challenged with *S. aureus* or *E. ictaluri*, the expression levels of *Pc-crustin 4* showed up-regulation trends at different degrees in the hemocytes, hepatopancreas, gills, and intestine tissues. Besides, the results of liquid antibacterial assay showed that r*Pc*-crustin 4 inhibited obviously the growth of *S. aureus* and *E. ictaluri*. The results of bacteria binding assay showed that r*Pc*-crustin 4 could bind strongly to *S. aureus* and *E. ictaluri*. Finally, RNAi assay was performed to study the immunity roles of *Pc-crustin 4* in crayfish *in vivo*. Taken together, *Pc-crustin 4* is an important immunity effector molecule, which plays crucial roles in defending against bacterial infection in crayfish.

1. Introduction

In invertebrates, the innate immunity system permits hosts to control, suppress or prohibit microbial growth shortly post the infection [1]. As the important effectors in the innate immunity system, antimicrobial peptides (AMPs) play important roles in systemic defenses against microbial invasion [2]. The main advantage of the AMPs used as the effectors in the innate immunity system is that high specificity is not indispensable [3]. AMPs usually act inhibitory activities on a broad spectrum of microorganisms. And they are a kind of small bioactive molecules, which are expressed in a wide range of taxa, from invertebrates to vertebrates, and from plants to animals [4]. AMPs are a kind of conserved peptide families which can play an important role in the innate immune system of invertebrates.

At present, a variety of AMP families have been reported in crustaceans (for example, shrimp and crarfish), including penaeidins [5–7], antilipopolysaccharide factors (ALFs) [8–10], lysozymes [11–13], and crustins [14–16]. More than 2000 AMPs have been reported in animals, according to the statistic results in the Antimicrobial Peptide Sequences Database [17]. As a kind of endogenous antibiotic materials, crustins mainly play the antimicrobial functions in the innate immunity systems of crustaceans. And there are also some research results about the antiviral functions of crustins [17–21]. The crustins family was firstly reported in *Carcinus maenas* with activity against Gram-positive bacteria [22]. Subsequently, quite a few cDNAs of crustins and crustins-like peptides have been identified from other crustaceans and Hymenoptera insect [23].

Crustins are cationic AMPs, which include three basic components: signal peptide, multi-domain region at N-terminus, whey acidic protein (WAP) domain at C-terminus [24]. The multi-domain region is mainly rich in a variety of amino acids, including glycine-rich, proline-rich, or cysteine-rich [24]. The WAP domain is composed of about 50 amino acids, including 8 cysteine residues which can form a 4-disulfide core (4-DSC) [17]. And it is the crucial structure element for the biological activity. In the aspect of structure classification, crustins are firstly classified into three major groups (type I-III) [25], but recently type IV

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and type V crustins was nominated [23]. Type I crustins have cysteine-rich region between the signal peptide and WAP domain. Type II crustins contain glycine-rich region at N-terminus followed by cysteine-rich region between the signal peptide and WAP domain. The Type III crustins include or lack a short proline and/or arginine-rich region at the N-terminus between the signal peptide and WAP domain [25]. The Type IV crustins have two WAP domains. The Type V crustins are similar to Type I crustins in structure. However, an extra aromatic amino acid-rich region exists between the cysteine-rich region and WAP domains [23].

At present, numerous crustins have been reported in crustaceans, which have antimicrobial activities against Gram-positive bacteria or Gram-negative bacteria. Besides, some crustins have proteinase inhibitory activities, for example, *Fenneropenaeus. chinensis* SWD [26], *P. clarkii* SWD [27], and *F. chinensis* DWD [28]. These results demonstrate the importance role of crustins in the immunity system of crustaceans. In this study, we identified a new crustin protein gene in fresh water crayfish, *Procambarus clarkii*, which was named as *Pc-crustin 4*. In the aspect of sequences analysis, amino acids sequences alignment and phylogenetic analysis were done. The normal tissue distribution and time course expression profiles were examined after the bacterial infection. The liquid antibacterial assay, bacteria binding assay, and RNAi assay were carried out, after recombinant expression and purification for r*Pc*-Crustin 4. The results showed that *Pc*-Crustin 4 is an important immunity effector in defending against bacterial infection in crayfish.

2. Materials and methods

2.1. Bacteria challenge and tissues collection

P. clarkii (about 15–20 g each) were bought from an aquatic market in Baotou city, Inner Mongolia autonomous region, China. They were cultured temporarily in laboratory tanks filled with fresh water for two weeks. And they were fed twice a day with artificial food during whole assay [29]. For bacteria-challenged experiment, *Staphylococcus aureus* or *Edwardsiella ictaluri* (2×10^7 cells per crayfish) was injected respectively into the abdominal segment of each crayfish [29]. Then hemolymph was taken from ventral sinus at different time points (2, 6, 12, 24, and 36 h) after bacterial challenge, using a 1 ml sterile syringe preloaded with 200 μl anticoagulant (10% sodium citrate, pH 7). Hemolymph was centrifuged immediately at 800 × *g* for 5 min (4 °C) to isolate the hemocytes [27]. Other tissues such as hepatopancreas, gills, and intestine were also collected at different time points (2, 6, 12, 24, and 36 h) after bacterial challenge for total RNA extraction.

Besides, hemolymph, hemocytes, and other three tissues (hepatopancreas, gills, and intestine) from normal crayfish were extracted using the same method. For normal crayfish, there was no treatment for crayfish. The injection of the same volume of sterile 1 × PBS was also used as a control. And three parallel experiments were done to improve the integrity of this work [29].

2.2. Total RNA extraction and cDNA synthesis

Total RNA of above-mentioned four tissues (hemocytes, hepatopancreas, gills, and intestine) from bacteria challenged and normal crayfish at different time points were extracted using RNAiso Plus DNase I (Promega, USA) according to the protocol. Then they were dissolved in DEPC treated water. And electrophoresis on 1% agarose gel free of RNase was done to test the quality of total RNA.

Next, first strand cDNA synthesis was done in 25 μl reaction volume containing 5 μg RNA, 1 μl M-MLV reverse transcriptase (Promega USA), 1 mM dNTP mixture using SMART F (5′-tac ggc tgc gag aag acg aca gaa ggg-3′), and Oligo anchor R (5′-gac cac gcg tat cga tgt cga $ct_{16}v$-3′) at 42 °C for 2 h [28]. At last, synthetic cDNA was stored at −80 °C refrigerator for gene cloning and expression patterns research.

2.3. Gene cloning

On the base of the specific nucleotide sequences obtained from our previous transcriptome sequencing, the specific forward and reverse primers (F1:5′-cgt acg aga gga atg tgc-3′; R1:5′-gca gag gaa cac ata tct tg-3′) were designed. The F1 and 3′ anchor R primer (5′-gac cac gcg tat cga tgt cga c-3′) were used to amplify the 3′ end of the target gene cDNA sequence. The 5′ PCR primer (5′-tac ggc tgc gag aag acg aca gaa-3′) and the R1 were used to amplify the 5′ end of the target gene cDNA sequence. Polymerase chain reaction (PCR) amplification was carried out as follows: a cycle of 94 °C for 3 min and 35 cycles of 94 °C for 30 s, 54 °C for 45 s, and 72 °C for 45 s, followed by an additional extension at 72 °C for 5 min. PCR products were purified using the gel purification kit (Sangon, China) according to the protocol, which was followed by the ligation into the pMD-18T vector (Takara, Japan) and transformed into the competent DH5α cells [17].

Then positive recombinants were identified through blue-white color selection in ampicillin-containing LB plates and the PCR screening using the two specific primers (F1 and R1), respectively [27]. At last, positive clones were sequenced by Sangon companies (Sangon, Shanghai, China).

2.4. Sequence alignment and phylogenetic analysis

After obtaining completed cDNA sequence of target gene, BLASTx online analysis was performed on the website (http://www.ncbi.nlm.nih.gov/). Translation of the amino acid sequences and prediction of the deduced protein were done with ExPASy online tool (http://www.expasy.org). Signal peptides sequence and structural domain were predicted using SMART (Simple Modular Architecture Research Tool) (http://www.smart.embl-heidelberg.de/) [27]. Amino acids sequences alignment with crustins homogenous sequences from other invertebrates, which were selected by the BLASTx analysis results, was done using MEGA 7.0 software. Phylogenetic analysis was carried out using Neighbor Joining (NJ) methods of MEGA 7.0 based on the amino acid sequences. To estimate the reliability, 1000 bootstraps were selected for the NJ tree [28].

2.5. Quantitative real-time PCR analysis for target gene expression patterns after bacteria challenge

Quantitative real-time PCR (qRT-PCR) analysis was performed in four tissues (hemocytes, hepatopancreas, gills, and intestine) of normal crayfish. And it was also carried out in the four corresponding tissues of bacteria challenged crayfish at different time points (2, 6, 12, 24, and 36 h after bacterial challenge). In brief, 5 μg total RNA from each tissue was used to reverse transcribe the first strand of cDNA, which was used as the template in PCR reactions. For qRT-PCR assay, cDNA templates were diluted 40-fold in nuclease-free water. A pair of primers (F2: 5′-gag aag tgc tgt tac ctc-3′; R2: 5′- gca gag gaa cac ata tct tg -3′) were used to amplify target gene fragment in qRT-PCR. The specific primers, *18S RNA*-RT-F(5′-tct tct tag agg gat tag cgg-3′)and *18S RNA*-RT-R(5′-aag ggg att gaa cgg gtt a-3′)were used to amplify corresponding *18S RNA* gene fragment as the inner control [29].

The qRT-PCR was performed following the manufacturer's instruction of SYBR Premix Ex Taq (Takara, Japan) using a real-time thermal cycler (Bio-Rad, USA) in a total volume of 20 μl containing 10 μl of 2 × Premix Ex Taq, 2 μl of the 1:40 diluted cDNA, and 4 μl (1 μM) each of the forward and reverse primer [17,29]. The amplification procedure consisted of an initial denaturation step at 95 °C for 3 min and then 40 cycles of 95 °C for 15 s, 56 °C for 30 s, followed by melting from 65 °C to 95 °C. In amplification process, the melting curve was analyzed for amplification products to confirm the uniqueness. And it was done at the end of each PCR reaction. Besides, three parallel experiments were carried out to ensure the integrity (we selected three different batches of crayfish to carry out immune challenge, and then extracted RNA, synthesized cDNA, performed qRT-PCR, respectively). Moreover, the expression level of target gene was shown as relative expression values, which were calculated according to $2^{-\Delta\Delta CT}$ method [3,4]. The data were subjected to statistical analysis followed by an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$. Extremely significant difference was accepted at $P < 0.01$.

2.6. Recombinant expression and purification

The mature peptide molecule was amplified by the expression primers (Exp-F: 5′-tac tca gaa ttc cgc tcc cca ccc ttc cct-3′, Exp-R: 5′-tac tca ctc gag tta ggc gtc ctc tga gtt-3′; *Eco*R I and *Xho* I sites were underlined). The recombinant expression vector was constructed and generated by subcloning corresponding mature cDNA into the *Eco*R I and *Xho* I sites of pET-28a. The constructed plasmid was transformed into competent cells of *E. coli* BL21(DE3) for recombinant expression. Overnight culture positive transformants (1 ml) were transferred into 100 ml kanamycin-containing Luria-Bertani broth for the large-scale culture. When the OD_{600} value was up to 0.6, the final concentration of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce recombinant protein expression. The induced temperature was 28 °C and the induced time was 14–16 h. At last, recombinant protein was purified with His Bind resin chromatography (Sangon, China) following the protocol [27].

When purified recombinant protein was used as antigen to produce polyclonal rabbit antiserum, a method described in a previous study was referenced [28]. The polyclonal rabbit antiserum was used to detect corresponding recombinant protein in subsequent assay.

2.7. Liquid antibacterial assay in vitro

To detect the antibacterial activity of purified recombinant protein, the liquid antibacterial assay was performed according to the method of antibacterial susceptibility testing in liquid media, which was recommended by the National Committee of Laboratory Safety and Standards [30]. The antibacterial activity was tested against *S. aureus* and *E. ictaluri*. In brief, the bacteria were cultured overnight, and washed twice with 1 × PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2). Then they were suspended in poor broth media (PB media: 1% tryptone, 0.5% NaCl (w/v)). The concentration of bacterial solution was regulated to about 10^5 cells per milliliter of media [30]. The purified recombinant protein, which was overnight dialyzed in 1 × PBS buffer, was concentrated and regulated to $0.2\,mg\,ml^{-1}$. Then recombinant protein solution (1 ml) was incubated with 2 ml bacteria solution in 5.0 ml eppendorf tube at 22 °C. Subsequently, the absorbance at 630 nm was measured by a plate reader, at 0, 2, 4, 6, 8, 10, 12, 24, and 36 h after incubation beginning. The 1 × PBS buffer was set as the control. The absorbance of control was normalized to 1, and others were showed by the relative concentrations of bacteria. The whole assay was independently repeated three times. And the data were subjected to statistical analysis followed by an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$. Extremely significant difference was accepted at $P < 0.01$.

2.8. Bacteria binding assay for purified recombinant protein

The binding assay to microorganism was performed respectively using Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. ictaluri*). In brief, the overnight cultured bacteria were pelleted by centrifugation at $6000 \times g$ for 5 min, washed with TBS buffer for three times, and thoroughly re-suspended in TBS buffer. The purified recombinant protein ($0.2\,mg\,ml^{-1}$, 400 μl) was incubated with 400 μl overnight cultured bacteria, and subjected to gentle rotation for 2 h at room temperature. Then bacteria were pelleted, and washed three times with TBS buffer. Subsequently, bacteria precipitate was subjected to elution with 7% SDS for 10 min, and washed for three times with 0.5 ml TBS buffer. At last, the washed bacteria were subjected to 15% SDS-PAGE. Besides, *Brucella suis* outer membrane protein 22 (Omp22), which was expressed by the same expression system, was used as a control [27]. The r*Bs*-Omp22 was detected by the rabbit antiserum against r*Bs*-Omp22, which was produced in our laboratory as well.

2.9. RNAi assay and crayfish survival rate detection

Double strand RNA (dsRNA) for target gene was synthesized using the methods described previously in another article [29]. In brief, crayfish was divided randomly into three groups (30 for each group), including *Pc-crustin* 4 dsRNA (*dsPc-crustin 4*) injection group, GFP dsRNA (*dsGFP*) injection group, and normal group. Normal crayfish did not receive any treatment. *Pc-crustin* 4 and GFP DNA fragments were amplified using *Pc-crustin* 4-Fi (5′-gcg taa tac gac tca cta tag gat gag gcg agt gtg tgt g-3′) and *Pc-crustin* 4-Ri (5′-gcg taa tac gac tca cta tag gtt agg cgt cct ctg agt tac -3′), GFP-Fi (5′-gcg taa tac gac tca cta tag gtg gtc cca att ctc gtg gaa c-3′) and GFP-Ri (5′-gcg taa tac gac tca cta tag gct tga agt tga cct tga tgc c-3′), respectively. The sequence of T7 promotor was underlined in the primers above. The DNA fragments obtained by PCR were used as templates for dsRNA synthesis. The dsRNA synthesis system was designed according to the protocol of *in vitro* Transcription T7 promotor kit (Takara, Japan). At last, dsRNA was extracted with phenol/chloroform and precipitated with ethanol, then resuspended in 40 μl RNase-Free water.

The prepared dsRNA (about 30 μg) was injected into the abdominal segment of each crayfish, and the second injection of dsRNA (about 30 μg) was given 24 h later to enhance the RNAi efficiency [29]. Subsequently, the total RNA was isolated from hemocytes of three groups' crayfish at 2 h after the second injection of dsRNA. Meantime, *S. aureus* or *E. ictaluri* (2×10^7 cells per crayfish) was injected respectively into the abdominal segment of each crayfish in each group [29]. The survival rates of crayfish in three groups were counted respectively at different time points (2, 6, 12, 24, 36, 48, and 72 h) after bacteria injection. To evaluate the knockdown efficiency of *Pc-crustin 4*, qRT-PCR was carried out using the primer F2 (5′-gag aag tgc tgt tac ctc-3′) and R2 (5′- gca gag gaa cac ata tct tg -3′). Crayfish injected with dsRNA against GFP, mock treated crayfish (crayfish challenged by 1 × PBS), and crayfish only challenged by *S. aureus* or *E. ictaluri* served as control. Besides, three parallel experiments were carried out to increase the integrity of experiments.

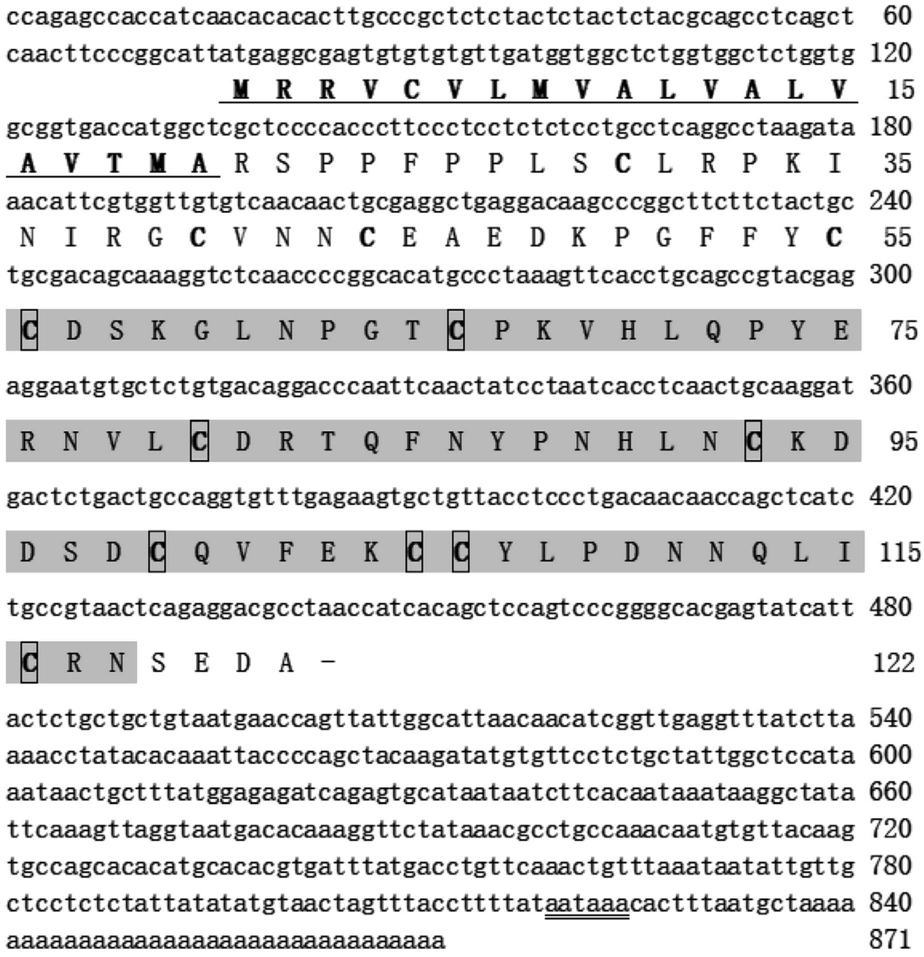
3. Results

3.1. Sequence cloning of Pc-crustin 4 cDNA

A total of 871 bp cDNA sequence was obtained after sequence splicing. The results obtained through BLAST showed that this sequence was highly homologous to the *P. clarkii crustin* gene family. According to the discovery order, it was nominated as *Pc-crustin 4*. The open reading frame (ORF) of *Pc-crustin 4* contained 369 bp, which encoded a protein of 122 amino acids, with a 20-amino-acid signal peptide sequence (SPS) located at the N-terminus. A classical WAP domain containing 8 conserved cysteines located at the C-terminus (Fig. 1). And a polyadenylation signal (aataaa) located 12 bp upstream of poly A tail. The mature peptide (102 amino acids) had a predicted molecular mass of 11.68 kDa and an estimated *p*I of 6.09. Besides, there were four additional cysteines between the SPS and WAP domain. On the base of the classification method established by Smith et al., *Pc*-crustin 4 belonged to Type I crustin [25].

3.2. Multiple alignments and phylogenetic analysis for Pc-crustin 4

According to the results of BLAST online analysis, some crustin molecules from crustaceans were chosen to perform sequences multiple alignment. For example, *Eriocheir sinensis* crustin 1 (EU183310.1),



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               M  R  R  V  C  V  L  M  V  A  L  V  A  L  V    15
gcggtgaccatggctcgctccccacccttccctcctctctcctgcctcaggcctaagata 180
A  V  T  M  A  R  S  P  P  F  P  P  L  S  C  L  R  P  K  I    35
aacattcgtggttgtgtcaacaactgcgaggctgaggacaagcccggcttcttctactgc 240
N  I  R  G  C  V  N  N  C  E  A  E  D  K  P  G  F  F  Y  C    55
tgcgacagcaaaggtctcaaccccggcacatgccctaaagttcacctgcagccgtacgag 300
C  D  S  K  G  L  N  P  G  T  C  P  K  V  H  L  Q  P  Y  E    75
aggaatgtgctctgtgacaggacccaattcaactatcctaatcacctcaactgcaaggat 360
R  N  V  L  C  D  R  T  Q  F  N  Y  P  N  H  L  N  C  K  D    95
gactctgactgccaggtgtttgagaagtgctgttacctccctgacaacaaccagctcatc 420
D  S  D  C  Q  V  F  E  K  C  C  Y  L  P  D  N  N  Q  L  I   115
tgccgtaactcagaggacgcctaaccatcacagctccagtcccggggcacgagtatcatt 480
C  R  N  S  E  D  A  -                                        122
actctgctgctgtaatgaaccagttattggcattaacaacatcggttgaggtttatctta 540
aaacctatacacaaattaccccagctacaagatatgtgttcctctgctattggctccata 600
aataactgctttatggagagatcagagtgcataataatcttcacaataaataaggctata 660
ttcaaagttaggtaatgacacaaaggttctataaacgcctgccaaacaatgtgttacaag 720
tgccagcacacatgcacacgtgatttatgacctgttcaaactgtttaaataatattgttg 780
ctcctctctattatatatgtaactagtttaccttttataataaacactttaatgctaaaa 840
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa                              871
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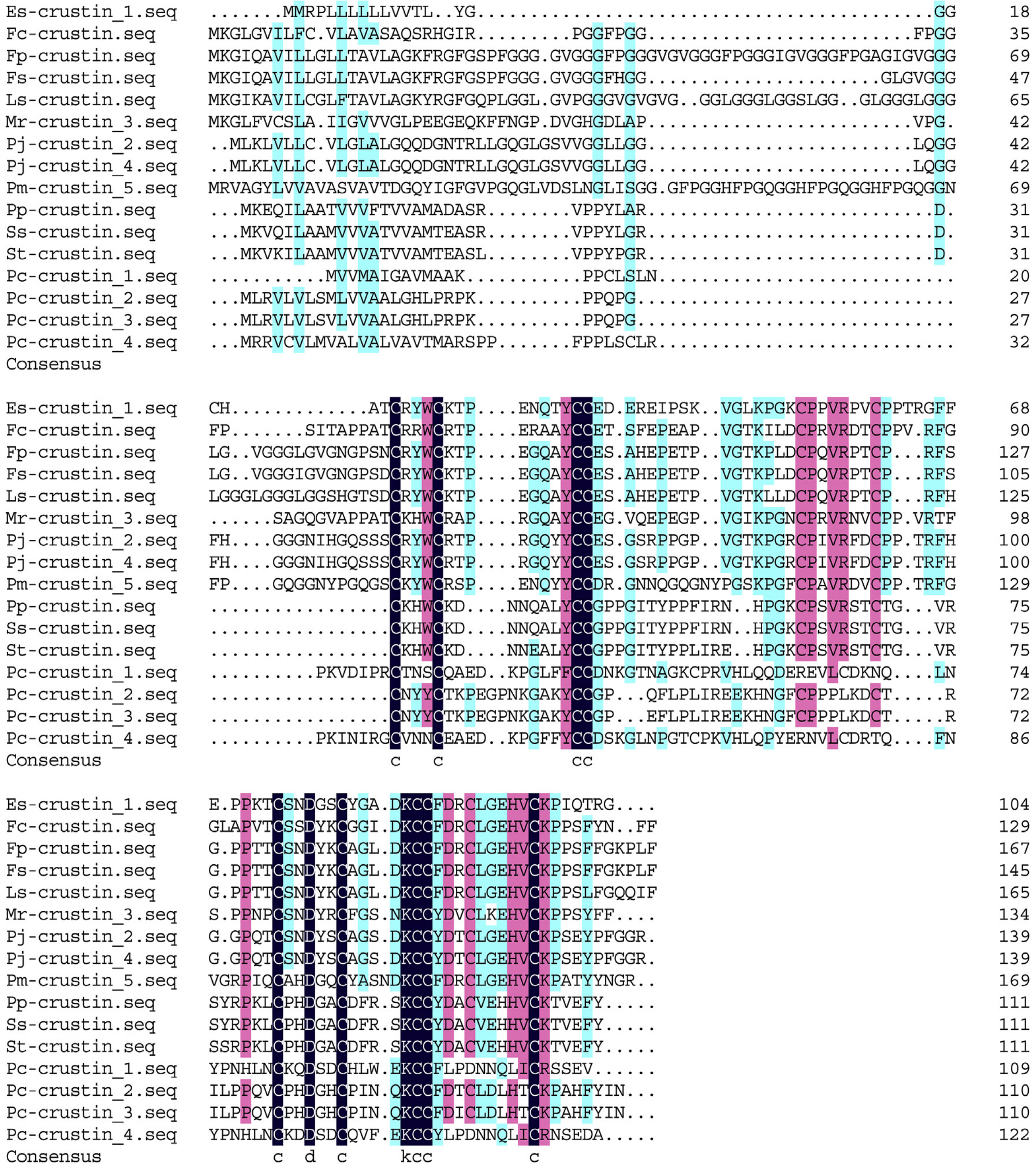
Fig. 1. Full length cDNA and deduced amino acids sequences of *Pc-crustin 4* from *P. clarkii*. The signal peptides are shown in bold letters and underlined. The whey acidic protein (WAP) domain is shaded in gray. The conserved cysteine residues are highlighted in bold and boxed.

Fenneropenaeus chinensis crustin (AY871268.1), *Farfantepenaeus paulensis* crustin (EF182747.1), *Farfantepenaeus subtilis* crustin (EF450744.1), *Litopenaeus schmitti* crustin (EF182748.1), *Macrobrachium rosenbergii* crustin 3 (KX219628.1), *Panulirus japonicus* crustin 2 (FJ797418.1), *Panulirus japonicus* crustin 4 (FJ797420.1), *Penaeus monodon* crustin 5 (FJ380049.1), *Portunus pelagicus* crustin (JQ965930.1), *Scylla serrata* crustin (HQ638025.1), *Scylla tranquebarica* crustin (JQ753312.1), *P. clarkii* crustin 1 (ACY64751.1), *P. clarkii* crustin 2 (ACY64752.1), *P. clarkii* crustin 3 (AEB54630.1). The alignment results showed that the sequences similarity was 36.97% among above-mentioned crustin molecules (Fig. 2). To further investigate the evolution relationship between *Pc*-crustin 4 and other chosen crustaceans crustin molecules, phylogenetic analysis was carried out. And the results showed that phylogenetic tree divided obviously into two clusters (Fig. 3). Those crustins, which were belonged to Type I crustin, included *Pp*-crustin, *Ss*-crustin, *St*-crustin, *Pc*-crustin 1, *Pc*-crustin 2, *Pc*-crustin 3. And those crustins, which were belonged to Type II crustin, included *Fc*-crustin, *Mr*-crustin, *Pj*-crustin 2, *Pj*-crustin 4, *Pm*-crustin, *Fs*-crustin, *Fp*-crustin, *Ls*-crustin. The *Pc*-crustin 4 located at the cluster of Type I crustin.

3.3. Expression profiles analysis of Pc-crustin 4 in mRNA level

In order to determine the expression patterns of *Pc-crustin 4*, total RNA was extracted respectively from four tissues (hemocytes, hepatopancreas, gills, and intestine) of normal crayfish, 1 × PBS-challenged crayfish, and bacteria-challenged crayfish. The results of qRT-PCR showed that *Pc-crustin 4* transcripts were expressed in hemocytes at relatively high level, and relatively low level in hepatopancreas, gills, and intestine in normal crayfish (Fig. 4A). Besides, the expression profiles of *Pc-crustin 4* were detected in hemocytes, hepatopancreas, gills, and intestine of normal crayfish, 1 × PBS-challenged crayfish, *S. aureus*-challenged crayfish, and *E. ictaluri*-challenged crayfish 12 h post-injection, respectively. In the mock-challenged (1 × PBS solution) crayfish, no obvious change was detected for the *Pc-crustin 4* expression. However, the expression levels of *Pc-crustin 4* were obviously up-regulated in the hemocytes, hepatopancreas, gills, and intestine of *S. aureus*-challenged crayfish, and *E. ictaluri*-challenged crayfish 12 h post-injection (Fig. 4B).

When challenged with *S. aureus*, the expression of *Pc-crustin 4* obviously increased from 2 to 24 hpi (hours post infection) and recovered to the normal level at 36 hpi in the hemocytes of crayfish. The expression level was obviously up-regulated to the maximum at 6 hpi, and there was almost a 5.5-fold increase (Fig. 5A). In the hepatopancreas of *S. aureus*-challenged crayfish, the expression level of *Pc-crustin 4* was up-regulated from 2 to 12 hpi, but recovered to the normal level from 24 to 36 hpi. The expression level increased to the maximum at 12 hpi, and there was almost a 4.2-fold increase (Fig. 5B). In the gills of *S. aureus*-challenged crayfish, the expression level of *Pc-crustin 4* was initially down-regulated at 2 hpi and obviously up-regulated from 6 to 24 hpi. The expression level increased to the maximum at 12 hpi, and there was almost a 3.2-fold increase (Fig. 5C). In the intestine of *S. aureus*-challenged crayfish, the expression level of *Pc-crustin 4* was up-regulated from 2 to 12 hpi and recovered to the normal level from 24 to 36 hpi. The expression level increased to the maximum at 12 hpi, and there was almost a 3.5-fold increase (Fig. 5D).



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Es-crustin_1.seq   .......MMRPLLLLLLVVTL..YG...........................................GG    18
Fc-crustin.seq     MKGLGVILFC.VLAVASAQSRHGIR.........PGGFPGG.........................FPGG    35
Fp-crustin.seq     MKGIQAVILLGLLTAVLAGKFRGFGSPFGGG.GVGGGFPGGGVGVGGGFPGGGIGVGGGFPGAGIGVGGG    69
Fs-crustin.seq     MKGIQAVILLGLLTAVLAGKFRGFGSPFGGG.GVGGGFHGG......................GLGVGGG    47
Ls-crustin.seq     MKGIKAVILCGLFTAVLAGKYRGFGQPLGGL.GVPGGGVGVGVG..GGLGGGLGGSLGG..GLGGGLGGG    65
Mr-crustin_3.seq   MKGLFVCSLA.IIGVVVGLPEEGEQKFFNGP.DVGHGDLAP.........................VPG.    42
Pj-crustin_2.seq   ..MLKLVLLC.VLGLALGQQDGNTRLLGQGLGSVVGGLLGG.........................LQGG    42
Pj-crustin_4.seq   ..MLKLVLLC.VLGLALGQQDGNTRLLGQGLGSVVGGLLGG.........................LQGG    42
Pm-crustin_5.seq   MRVAGYLVVAVASVAVTDGQYIGFGVPGQGLVDSLNGLISGG.GFPGGHFPGQGGHFPGQGGHFPGQGGN    69
Pp-crustin.seq     ...MKEQILAATVVVFTVVAMADASR........VPPYLAR...........................D.    31
Ss-crustin.seq     ...MKVQILAAMVVVATVVAMTEASR........VPPYLGR...........................D.    31
St-crustin.seq     ...MKVKILAAMVVVATVVAMTEASL........VPPYPGR...........................D.    31
Pc-crustin_1.seq   ...........MVVMAIGAVMAAK...........PPCLSLN............................    20
Pc-crustin_2.seq   ...MLRVLVLSMLVVAALGHLPRPK..........PPQPG..............................    27
Pc-crustin_3.seq   ...MLRVLVLSVLVVAALGHLPRPK..........PPQPG..............................    27
Pc-crustin_4.seq   ...MRRVCVLMVALVALVAVTMARSPP.......FPPLSCLR............................    32
Consensus

Es-crustin_1.seq   CH.............ATCRYWCKTP....ENQTYCCED.EREIPSK..VGLKPGKCPPVRPVCPPTRGFF    68
Fc-crustin.seq     FP.......SITAPPATCRRWCRTP....ERAAYCCET.SFEPEAP..VGTKILDCPRVRDTCPPV.RFG    90
Fp-crustin.seq     LG..VGGGLGVGNGPSNCRYWCKTP....EGQAYCCES.AHEPETP..VGTKPLDCPQVRPTCP...RFS   127
Fs-crustin.seq     LG..VGGGIGVGNGPSDCRYWCKTP....EGQAYCCES.AHEPETP..VGTKPLDCPQVRPTCP...RFS   105
Ls-crustin.seq     LGGGLGGGLGGSHGTSDCRYWCKTP....EGQAYCCES.AHEPETP..VGTKLLDCPQVRPTCP...RFH   125
Mr-crustin_3.seq   ......SAGQGVAPPATCKHWCRAP....RGQAYCCEG.VQEPEGP..VGIKPGNCPRVRNVCPP.VRTF    98
Pj-crustin_2.seq   FH....GGGNIHGQSSSCRYWCRTP....RGQYYCCES.GSRPPGP..VGTKPGRCPIVRFDCPP.TRFH   100
Pj-crustin_4.seq   FH....GGGNIHGQSSSCRYWCRTP....RGQYYCCES.GSRPPGP..VGTKPGRCPIVRFDCPP.TRFH   100
Pm-crustin_5.seq   FP....GQGGNYPGQGSCKYWCRSP....ENQYYCCDR.GNNQGQGNYPGSKPGFCPAVRDVCPP.TRFG   129
Pp-crustin.seq     .................CKHWCKD....NNQALYCCGPPGITYPPFIRN..HPGKCPSVRSTCTG...VR    75
Ss-crustin.seq     .................CKHWCKD....NNQALYCCGPPGITYPPFIRN..HPGKCPSVRSTCTG...VR    75
St-crustin.seq     .................CKHWCKD....NNEALYCCGPPGITYPPLIRE..HPGKCPSVRSTCTG...VR    75
Pc-crustin_1.seq   ..........PKVDIPRCTNSCQAED..KPGLFFCCDNKGTNAGKCPRVHLQQDEREVLCDKNQ....LN    74
Pc-crustin_2.seq   .................CNYYCTKPEGPNKGAKYCCGP...QFLPLIREEKHNGFCPPPLKDCT.....R    72
Pc-crustin_3.seq   .................CNYYCTKPEGPNKGAKYCCGP...EFLPLIREEKHNGFCPPPLKDCT.....R    72
Pc-crustin_4.seq   ..........PKINIRGCVNNCEAED..KPGFFYCCDSKGLNPGTCPKVHLQPYERNVLCDRTQ....FN    86
Consensus                           c   c            cc

Es-crustin_1.seq   E.PPKTCSNDGSCYGA.DKCCFDRCLGEHVCKPIQTRG....                               104
Fc-crustin.seq     GLAPVTCSSDYKCGGI.DKCCFDRCLGEHVCKPPSFYN..FF                               129
Fp-crustin.seq     G.PPTTCSNDYKCAGL.DKCCFDRCLGEHVCKPPSFFGKPLF                               167
Fs-crustin.seq     G.PPTTCSNDYKCAGL.DKCCFDRCLGEHVCKPPSFFGKPLF                               145
Ls-crustin.seq     G.PPTTCSNDYKCAGL.DKCCFDRCLGEHVCKPPSLFGQQIF                               165
Mr-crustin_3.seq   S.PPNPCSNDYRCFGS.NKCCYDVCLKEHVCKPPSYFF....                               134
Pj-crustin_2.seq   G.GPQTCSNDYSCAGS.DKCCYDTCLGEHVCKPSEYPFGGR.                               139
Pj-crustin_4.seq   G.GPQTCSNDYSCAGS.DKCCYDTCLGEHVCKPSEYPFGGR.                               139
Pm-crustin_5.seq   VGRPIQCAHDGQCYASNDKCCFDRCLGEHVCKPATYYNGR..                               169
Pp-crustin.seq     SYRPKLCPHDGACDFR.SKCCYDACVEHHVCKTVEFY.....                               111
Ss-crustin.seq     SYRPKLCPHDGACDFR.SKCCYDACVEHHVCKTVEFY.....                               111
St-crustin.seq     SSRPKLCPHDGACDFR.SKCCYDACVEHHVCKTVEFY.....                               111
Pc-crustin_1.seq   YPNHLNCKQDSDCHLW.EKCCFLPDNNQLICRSSEV......                               109
Pc-crustin_2.seq   ILPPQVCPHDGHCPIN.QKCCFDTCLDLHTCKPAHFYIN...                               110
Pc-crustin_3.seq   ILPPQVCPHDGHCPIN.QKCCFDICLDLHTCKPAHFYIN...                               110
Pc-crustin_4.seq   YPNHLNCKDDSDCQVF.EKCCYLPDNNQLICRNSEDA.....                               122
Consensus                c  d  c     kcc         c
```

Fig. 2. Amino acids sequences alignment of *Pc*-Crustin 4 with other Crustins. The numbers on the right indicated the amino acid position of different sequences. Different colors represented the different conservations of amino acids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

When crayfish was challenged with *E. ictaluri*, the obvious up-regulation trends of *Pc-crustin 4* expression level also appeared in the hemocytes, hepatopancreas, gills, and intestine tissues. In the *E. ictaluri*-challenged hemocytes, the expression level of *Pc-crustin 4* was up-regulated from 2 to 12 hpi and increased to the maximum at 6 hpi with a 4.7-fold increase. Then the expression level recovered to the normal level from 24 to 36 hpi (Fig. 6A). In the *E. ictaluri*-challenged hepatopancreas, the expression level of *Pc-crustin 4* was also up-regulated from 2 to 12 hpi and increased to the maximum at 6 hpi with a 3.4-fold increase. Then the expression level recovered to the normal level from 24 to 36 hpi (Fig. 6B). In the *E. ictaluri*-challenged gills, the expression level of *Pc-crustin 4* was initially down-regulated at 2 hpi and obviously up-regulated from 6 to 24 hpi. The expression level increased to the maximum at 12 hpi, and there was almost a 3.2-fold increase (Fig. 6C). In the *E. ictaluri*-challenged intestine, the expression level of *Pc-crustin 4* was up-regulated from 2 to 12 hpi and recovered to the normal level from 24 to 36 hpi. The expression level increased to the maximum at 6 hpi, and there was almost a 3.1-fold increase (Fig. 6D).

3.4. Recombinant expression and purification

After positive expression strain was induced by 0.5 mM IPTG, the recombinant protein was highly expressed. The predicted molecular weight of *Pc*-crustin 4 was about 11.68 kDa. An extra His-tag fragment was expressed by the pET-28a plasmid in the N-terminal of the expressed fusion protein. The apparent molecular mass of the recombinant protein (r*Pc*-crustin 4) was about 17.28 kDa, which was consistent with the expected value (Fig. 7). Following the manufacturer's protocol, r*Pc*-crustin 4 was purified by His Bind resin chromatography (Sangon, China) and was used for the rabbit antiserum

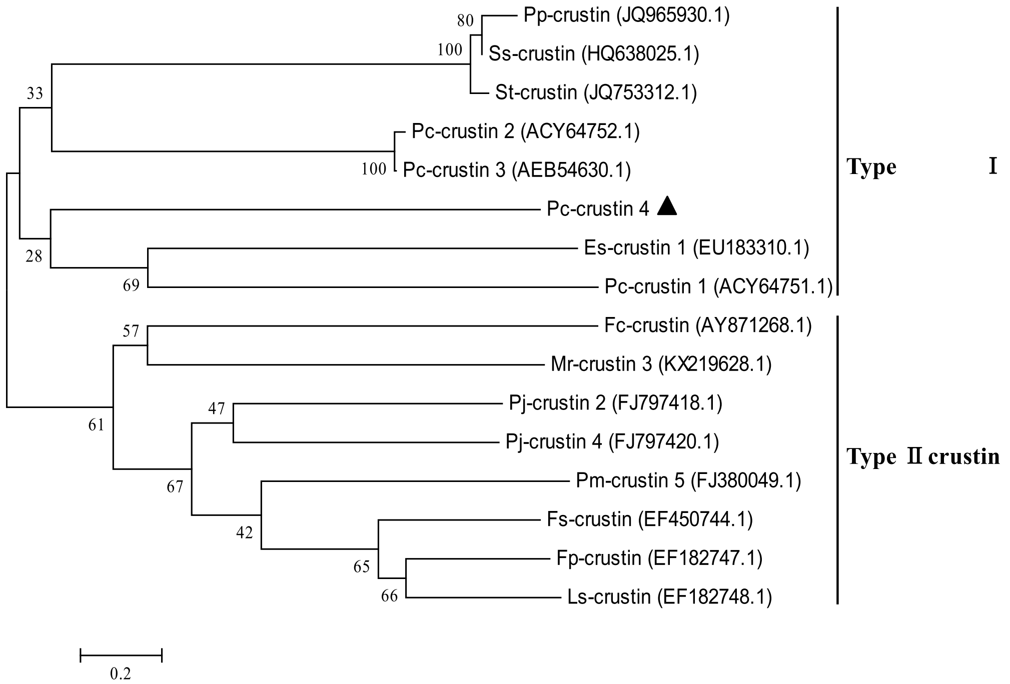

Fig. 3. Phylogenetic analysis of *Pc*-Crustin 4 with other known Crustins from crustaceans based on amino acids sequences. The neighbor-joining tree was constructed by molecular evolutionary genetics analysis (MEGA) software version 7.0. GenBank accession numbers followed the taxon names. *Pc*-Crustin 4 was showed in black triangle.

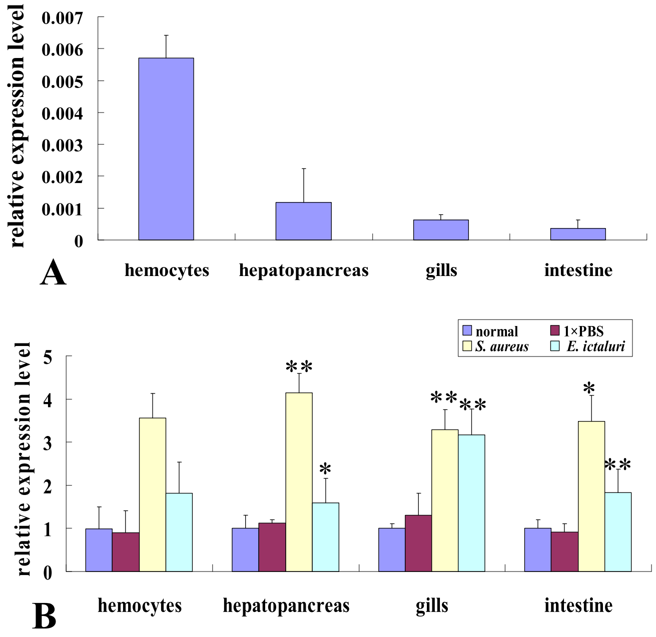

Fig. 4. Tissue distribution of *Pc-crustin 4* in normal tissues of crayfish (A) and expression profiles of *Pc-crustin 4* in hemocytes, hepatopancreas, gills, and intestine of normal crayfish, 1 × PBS-challenged crayfish, *S. aureus*-challenged crayfish, and *E. ictaluri*-challenged crayfish 12 h post-injection, respectively (B). The transcripts of *Pc-crustin 4* were tested by qRT-PCR. Expression profiles were showed in relative level to *18S rRNA* inner control gene. The expression level of *Pc-crustin 4* was shown as relative expression values, which were calculated according to $2^{-\Delta\Delta CT}$ method. Asterisks indicated the significant differences from the control (*: $P < 0.05$, **: $P < 0.01$). Error bars represented ± SD of 3 independent assays.

preparation and subsequent assays.

3.5. Antibacterial activity of rPc-crustin 4

After r*Pc*-crustin 4 was induced and expressed in *E. coli*, the liquid antibacterial assay was performed to detect whether it possessed antimicrobial activity *in vitro*. After incubation with r*Pc*-crustin 4 at 22 °C in poor broth media, the absorbance of *S. aureus* or *E. ictaluri* solution was tested at different time point post incubation, respectively. As shown in Fig. 8, r*Pc*-crustin 4 had obvious antimicrobial activity against *S. aureus* and *E. ictaluri*. Starting from 6 h post incubation, the growth rate of *S. aureus* slowed down significantly, compared with the control (Fig. 8A). And the difference appeared extremely significant difference at the subsequent time point. Meanwhile, the growth rate of *E. ictaluri* slowed down significantly at 8 h post incubation, compared with the control (Fig. 8B). The obvious inhibition trend continued until 36 h post incubation.

3.6. Bacterial binding activity of rPc-crustin 4

To further confirm the potential mechanism of antibacterial activities of r*Pc*-crustin 4, bacterial binding assay was performed (see materials and methods). The bacteria after elution with 7% SDS (strong binding) were collected to run SDS-PAGE, and the bound r*Pc*-crustin was detected with the corresponding antibody by Western blotting (Fig. 9). Results showed that r*Pc*-crustin 4 could bind strongly to *S. aureus* (Gram-positive bacteria) and *E. ictaluri* (Gram-negative bacteria). However, *B. suis* outer membrane protein 22 (r*Bs*-Omp 22), which was expressed by the same expression system in this assay, bound to neither *S. aureus* nor *E. ictaluri*.

3.7. Survival rate of bacteria-challenged crayfish after rPc-crustin 4 was knocked down

To reveal the *in vivo* function of endogenous *Pc-crustin 4* during

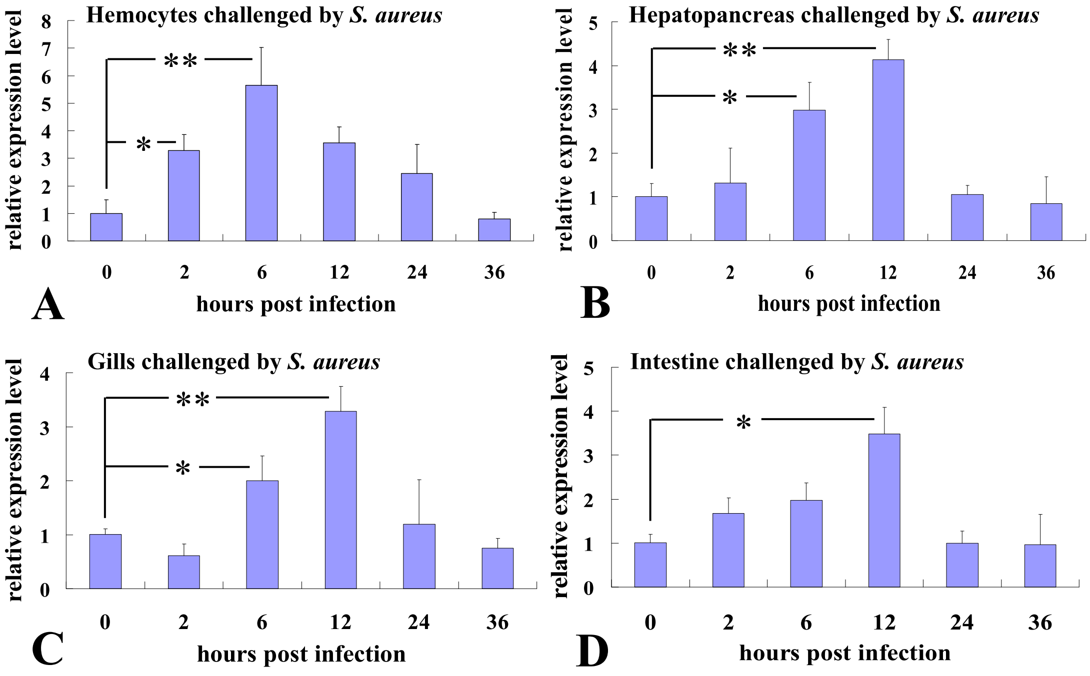

Fig. 5. Time course expression profiles of *Pc-crustin 4* in crayfish after challenged with *S. aureus*. Expression profiles of *Pc-crustin 4* in hemocytes (A), hepatopancreas (B), gills (C), and intestine (D) of crayfish were showed in relative level to *18S rRNA* inner control gene. Asterisks indicated the significant differences from the control (*: $P < 0.05$, **: $P < 0.01$). Error bars represented ± SD of 3 independent assays.

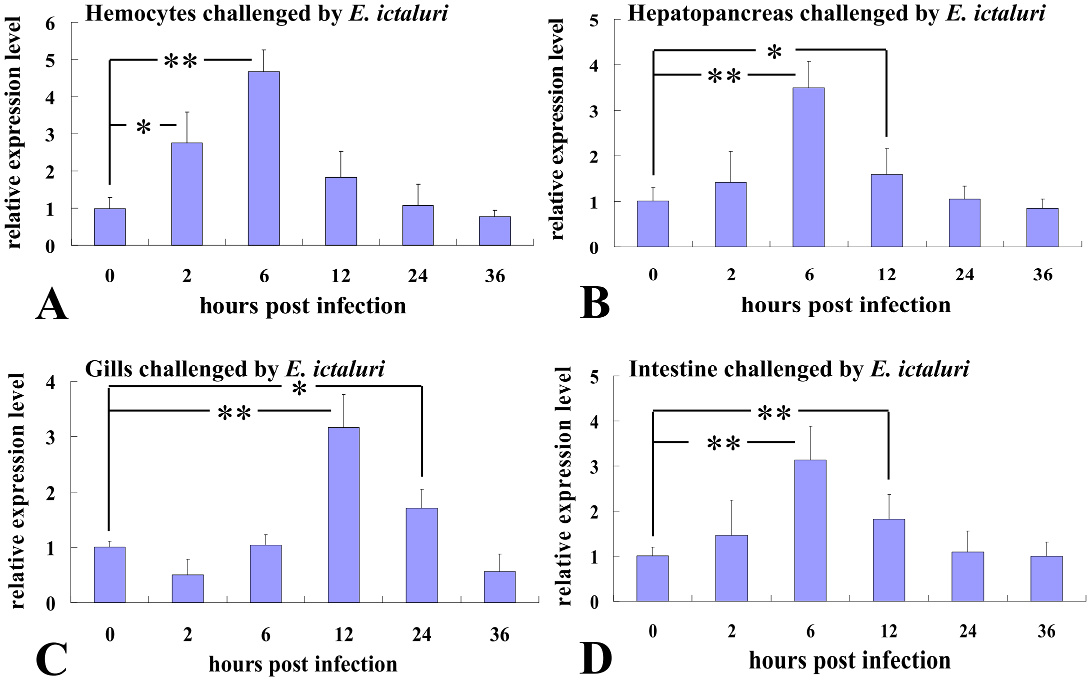

Fig. 6. Time course expression profiles of *Pc-crustin 4* in crayfish after challenged with *E. ictaluri*. Expression profiles of *Pc-crustin 4* in hemocytes (A), hepatopancreas (B), gills (C), and intestine (D) of crayfish were showed in relative level to *18S rRNA* inner control gene. Asterisks indicated the significant differences from the control (*: $P < 0.05$, **: $P < 0.01$). Error bars represented ± SD of 3 independent assays.

bacterial infection, RNAi assay was carried out to check whether it could play a crucial role in the antibacterial response. Two hours after the second dsRNA injection, total RNA was extracted from the crayfish hemocytes of three groups to detect the expression level of *Pc-crustin 4*. The results indicated that *Pc-crustin 4* was successfully knocked down compared with the *dsGFP* injection group and normal group (Fig. 10A).

After *S. aureus* and *E. ictaluri* were respectively injected into the crayfish abdominal segment of three groups, the number of survival crayfish at different time point was calculated. The results showed that the number of surviving crayfish decreased obviously after *S. aureus* (Fig. 10B) or *E. ictaluri* (Fig. 10C) was injected, compared with other three groups. These results suggested that endogenous *Pc-crustin 4* played an important role in the antibacterial innate immunity of crayfish.

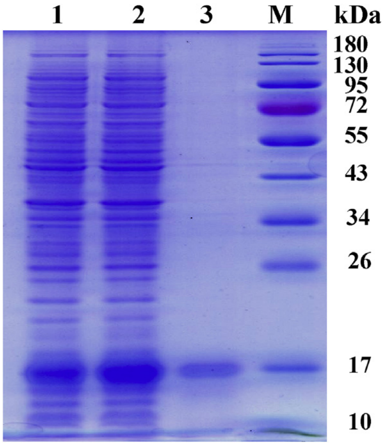

Fig. 7. SDS-PAGE analysis of recombinant *Pc*-Crustin 4 expressed with a His-tag in *E. coli*. Lane 1, total protein obtained from *E. coli* without induction; lane 2, total protein obtained from *E. coli* with IPTG induction; lane 3, recombinant *Pc*-Crustin 4 purified with His Bind resin chromatography; Lane M, protein marker.

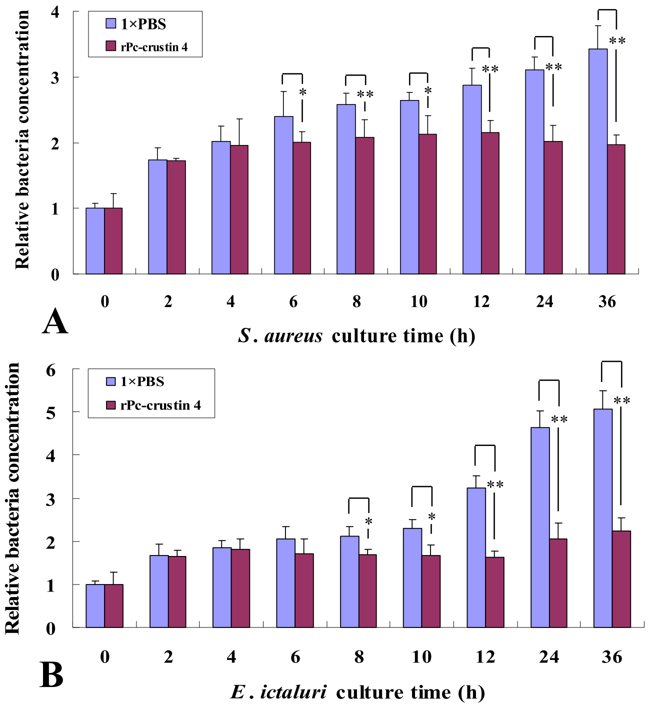

Fig. 8. The results of liquid antibacterial assay for r*Pc*-Custin 4. (A) The results of liquid antibacterial assay for r*Pc*-custin 4 against *S. aureus*. (B) The results of liquid antibacterial assay for r*Pc*-custin 4 against *E. ictaluri*. The absorbance at 630 nm was measured by a plate reader at 0, 2, 4, 6, 8, 10, 12, 24, and 36 h after incubation beginning. The 1 × PBS buffer was set as the control. The absorbance of control was normalized to 1, and others were showed by the relative concentrations of bacteria. Significant difference was accepted at $P < 0.05$. Extremely significant difference was accepted at $P < 0.01$.

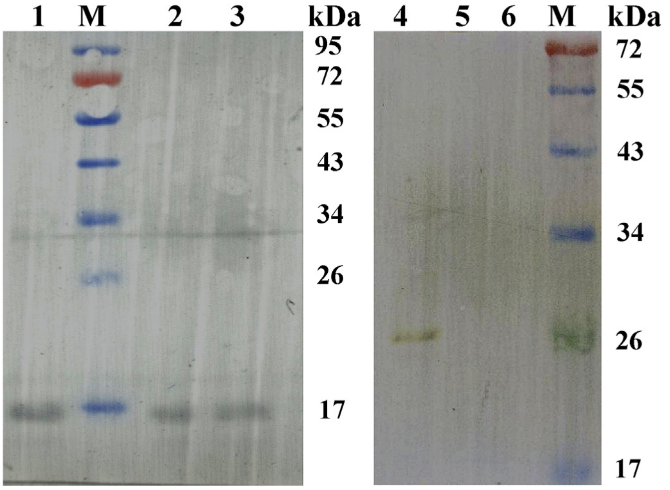

Fig. 9. Direct binding assay of r*Pc*-Crustin 4 to bacteria. The microorganisms binding assay of r*Pc*-Crustin 4 was carried out using a Gram-positive bacterium (*S. aureus*) and a Gram-negative bacterium (*E. ictaluri*). The protein bands were recognized by the antiserum against r*Pc*-DDC (1, 2, 3) or r*Bs*-Omp 22 (4, 5, 6) in western bolt. Lane 1, r*Pc*-Crustin 4; lane 2, final pellet fractions of *S. aureus*; lane 3, final pellet fractions of *E. ictaluri*; lane 4, r*Bs*-Omp 22; lane 5, final pellet fractions of *S. aureus*; lane 6, final pellet fractions of *E. ictaluri*; lane M, protein marker.

4. Discussion

In the past twenty years, some crustins molecules, which are important AMPs in the innate immunity system of invertebrates, have been identified in different crustaceans [17]. The first member of crustins family was reported in crab *C. maenas* in 1999, which was named as carcinin [22]. And it was an 11.5 kDa AMPs with the inhibitory activity against Gram-positive bacteria. Subsequently, some crustins and crustins-like genes were identified in the different crustaceans, on the base of the progress of gene sequencing technology [3,14–21,24,26–28]. In present study, we reported a new crustins gene in *P. clarkia*, which was named as *Pc-crustin 4*. The sequence full length of *Pc-crustin 4* is composed of 871 bp. And the ORF contains 369 bp which encodes a protein of 122 amino acids (Fig. 1).

In the molecular structure, a 20-amino-acid SPS locates at the N-terminus of *Pc*-crustin 4. And a typical WAP domain, which is mainly composed of 8 conserved cysteines, locates at the C-terminus (Fig. 1). Besides, there were 4 cysteines (C) residues between SPS and WAP domain in *Pc*-crustin 4. At the same time, there are also 6 prolines (P) and 3 arginines (R) between SPS and WAP domain in *Pc*-crustin 4. According to the latest classification method, *Pc*-crustin 4 should belong to Type-I or Type-III crustins [23]. These features indicate *Pc*-crustin 4 is a new type of crustins molecule. Combination with the results of phylogenetic tree analysis, we think that *Pc*-crustin 4 belongs to Type-I crustins (Fig. 3).

To study the immunity roles of *Pc-crustin 4* in crayfish *in vivo*, tissues distribution and time course expression patterns after bacteria infection were examined. The results of normal tissues distribution show that *Pc-crustin 4* transcripts are expressed in hemocytes at relatively high level, and relatively low level in hepatopancreas, gills, and intestine in normal crayfish (Fig. 4A). After respectively challenged with *S. aureus* or *E. ictaluri*, the expression levels of *Pc-crustin 4* show up-regulation trends at different degrees in the hemocytes, hepatopancreas, gills, and intestine of crayfish (Figs. 5 and 6). These results demonstrate that *Pc-crustin 4* responds to the *S. aureus* or *E. ictaluri* infection. And *Pc-crustin 4* should be an important innate immunity-related gene in crayfish.

At present, many crustins have been identified in crustaceans, which have antimicrobial activities against Gram-positive bacteria or Gram-negative bacteria [3,16,22,24,26,31–33]. Besides, some crustins have the proteinase inhibitory activities [27,28,30,34,35]. And some crustins are correlated with the defense against virus infection [18–21,36,37]. The various functions of crustins demonstrate the importance in crustaceans' immunity system. In this study, the results of liquid antibacterial assay show that r*Pc*-crustin 4 inhibits obviously the growth of *S. aureus* (Fig. 8A) and *E. ictaluri* (Fig. 8B). The results of bacteria binding assay show that r*Pc*-crustin 4 could bind strongly to *S. aureus* (Gram-positive bacteria) and *E. ictaluri* (Gram-negative bacteria) (Fig. 9). These results of two above-mentioned experiments

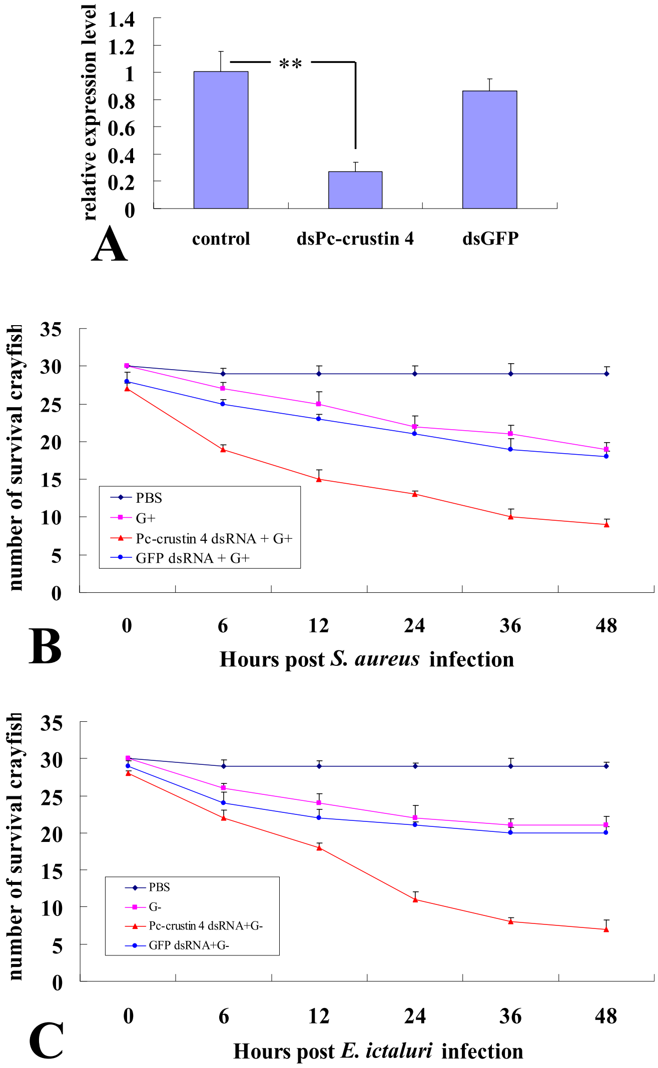

Fig. 10. RNAi assay was carried out to validate the role of *Pc-crustin 4* in crayfish antibacterial innate immune. (A) qPCR indicated that injection dsRNA against *Pc-crustin 4* knock down obviously the transcription of *Pc-crustin* 4 mRNA in hemocytes of crayfish. *18S RNA* was used as inner control. (B) Knockdown of *Pc-crustin 4* obviously decreased the surviving rate of crayfish after challenged by *S. aureus*. (C) Knockdown of *Pc-crustin 4* obviously decreased the surviving rate of crayfish after challenged by *E. ictaluri*. Crayfish injected with dsRNA against GFP, mock treated crayfish, and crayfish only challenged by *S. aureus* or *E. ictaluri* served as control.

demonstrate that r*Pc*-crustin 4 acts important roles in defending bacterial infections *in vitro*.

To study the functions of endogenous *Pc-crustin 4 in vivo* during bacterial infection, RNAi assay was done. The results show that the surviving rates of crayfish decrease obviously after *S. aureus* (Fig. 10B) or *E. ictaluri* (Fig. 10C) injection, when the expression level of *Pc-crustin* 4 mRNA is knocked down. These results reveal that endogenous *Pc-crustin 4* plays an important role *in vivo* in the antibacterial innate immunity of crayfish. Taken together, *Pc-crustin 4* is an important immunity effector molecule, which plays crucial roles in defending against the infections of pathogenic microorganisms in crayfish.

Acknowledgments

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