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A new crustin homologue (*Sp*Crus6) involved in the antimicrobial and antiviral innate immunity in mud crab, *Scylla paramamosain*

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ABSTRACT

Crustins play important roles in defending against bacteria in the innate immunity system of crustaceans. In present study, we identified a crustin gene in *Scylla paramamosain*, which was named as *SpCrus6*. The ORF of *Sp*Crus6 possessed a signal peptide sequence (SPS) at the N-terminus and a WAP domain at the C-terminus. And there were 5 Proline residues, 5 Glycine and 4 Cysteine residues between SPS and WAP domain in *Sp*Crus6. These features indicated that *Sp*Crus6 was a new member of crustin family. The *SpCrus6* mRNA transcripts were up-regulated obviously after bacteria or virus challenge. These changes showed that *SpCrus6* was involved in the antimicrobial and antiviral responses of *Scylla paramamosain*. Recombinant *Sp*Crus6 (r*Sp*Crus6) showed strong inhibitory abilities against Gram-positive bacteria (*Bacillus megaterium*, *Staphylococcus aureus*, and *Bacillus subtilis*). But the inhibitory abilities against four Gram-negative bacteria (*Vibrio parahemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi* and *Escherichia coli*) and two fungi (*Pichia pastoris* and *Candida albicans*) were not strong enough. Besides, r*Sp*Crus6 could strongly bind to two Gram-positive bacteria (*B. subtilis* and *B. megaterium*) and three Gram-negative bacteria (*V. alginolyticus*, *V. parahemolyticus*, and *V. harveyi*). And the binding levels to *S. aureus* and two fungi (*P. pastoris* and *C. albicans*) were weak. The polysaccharides binding assays' results showed r*Sp*Crus6 had superior binding activities to LPS, LTA, PGN and β-glucan. Through agglutinating assays, we found r*Sp*Crus6 could agglutinate well three Gram-positive bacteria (*S. aureus*, *B. subtilis* and *B. megaterium*). And the agglutinating activities to Gram-negative bacteria and fungi were not found. In the aspect of antiviral functions, r*Sp*Crus6 could bind specifically to the recombinant envelop protein 26 (rVP26) of white spot syndrome virus (WSSV) but not to recombinant envelop protein 28 (rVP28), whereas GST protein could not bind to rVP26 or rVP28. Besides, r*Sp*Crus6 could suppress WSSV reproduction to some extent. Taken together, *Sp*Crus6 was a multifunctional immunity effector in the innate immunity defending response of *S. paramamosain*.

1. Introduction

The mud crab *Scylla paramamosain* mainly distributes in the coasts of southeast China and other Asian countries [1]. Because of the short growth cycle, it has become an important economic aquaculture animal in China [2]. At the same time, it is also a good research material for innate immunity system of invertebrates. Unlike vertebrates, crustaceans merely have innate immunity system to defend against pathogens, including bacteria and virus [3]. As is known to all, innate immunity system is composed of cellular immunity and humoral immunity, mainly including phagocytosis, encapsulation, coagulation, melanization, and production of antimicrobial peptides (AMPs) [4,5].

Over the past few decades, many AMPs have been identified in different decapods crustaceans, in succession. For example, *Carcinus maenas* [6], *Penaeus monodon* [7], *Litopenaeus vannamei* [8], *Homarus gammarus* [9], *Fenneropenaeus chinensis* [10], *Marsupenaeus japonicus* [11], *Pacifastacus leniusculus* [12], *Procambarus clarkii* [13]. And several AMPs families have been confirmed, including penaeidin, antilipopolysaccharide factors (ALFs), lysozymes, and crustins [3]. The penaeidin family proteins possessed both Gram-positive antibacterial and

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antifungal activities, and the first penaeidin was found in *L. vannamei* [14]. ALFs which could bind to lipopolysaccharide (LPS) showed strong antimicrobial activity against Gram-negative bacteria [15]. And the first ALF was identified in the horseshoe crab *Limulus polyphemus* with strong antibacterial activities against Gram-negative bacteria [16]. Lysozymes were a kind of important immunity effectors in the innate immunity system of invertebrates, which included four types: c-type, g-type, i-type, and ch-type [17]. And it was first described in 1922 by Fleming [18]. The crustin family proteins which were initially described in *C. maenas* had strong inhibitory activity against Gram-positive bacteria [19].

To date, over 2000 AMPs have been reported in animals, on the base of the statistic results in the Antimicrobial Peptide Sequences Database (http://aps.unmc.edu/AP/main.php). AMPs belong to key elements of humoral immunity in the invertebrate's innate immunity system, and play an important role in defending and cleaning pathogens [20]. As a kind of endogenous antibiotic substance in crustaceans, AMPs are either constitutively expressed or induced by extraneous pathogens to play crucial roles in defending against bacteria and virus [21]. On the molecular structure, crustins belong to the WAP domain-containing AMPs [22]. The WAP domain is composed of about 50 amino acids, including 8 conserved cysteine residues which form a 4-disulfide core (4-DSC) [3]. It should be pointed out that the WAP domain is the key structure base for playing biological activity [23]. Besides, the sequence which locates between signal peptide sequences (SPS) and WAP domain is very important for WAP domain-containing AMPs' functions.

At present, numerous crustins have been identified in crustaceans, which had antimicrobial activities against Gram-positive bacteria or Gram-negative bacteria. Besides, some crustins possessed proteinase inhibitory activities, for example, *F. chinensis* SWD [24], *P. clarkii* SWD [3], and *F. chinensis* DWD [25]. The various functions demonstrate crustins' importance in crustaceans' immunity system. Up to now, five crustins exhibiting different antibacterial activities have been characterized in mud crab, *S. paramamosain* [26–29]. In present study, another new crustin homologue was identified in the same species, which showed low similarities with the other five reported crustins and was nominated as *SpCrus6*. After obtaining the cDNA sequence, amino acids sequences alignment and phylogenetic analysis were performed. The time course expression profiles were examined in gill after the infection of white spot syndrome virus (WSSV) or bacteria. After recombinant expression and purification for *Sp*Crus6, antimicrobial assay, microorganism-binding assay, microbial cell wall polysaccharides binding assay, and agglutinating assay were carried out. Besides, WSSV proliferation patterns and interactions between r*Sp*Crus6 and viral proteins were tested. The results showed that *Sp*Crus6 is a crucial immunity effector which plays an important role in defending against pathogens' infection.

2. Materials and methods

2.1. Reagents and chemicals

RNAiso Plus, First-Strand cDNA Synthesis Kit, and Taq Polymerase were purchased from TaKaRa Biotech (Dalian, China). Bacterial cell wall polysaccharides, including lipopolysaccharide (LPS, from *Escherichia coli* 0111: B4), lipoteichoic acid (LTA, from *Staphylococcus aureus*), peptidoglycan (PGN) from *S. aureus*, PGN from *Bacillus subtilis* (similar with Gram-negative bacterial PGN), and *β*-glucan from *Laminaria digitata*, were purchased from Sigma (St. Louis, MO, USA).

2.2. Mud crab challenge and tissue collection

Mud crabs *S. paramamosain* bought from a farm in Chongming County (Shanghai, China) were cultured in aerated seawater in 400 L tanks for a week prior to the experiments start. Animal experiments in this study were carried out in accordance with the international, national and institutional rules. The tissue distribution of *SpCrus6* was examined with normal individuals. The temporal expression patterns of *SpCrus6* were investigated after a respective challenge with 50 μL of *Vibrio parahemolyticus* (2×10^7 CFU), or 50 μL of *S. aureus* (2×10^8 CFU) suspension, or 100 μL of white spot syndrome virus (WSSV) inoculum by injecting into the base of the right fifth leg of each mud crab [30]. The corresponding control was challenged with 50 μL of sterile PBS (140 mM NaCl and 10 mM sodium phosphate, pH 7.4) or the supernatant of ground normal tissues. After the crabs were placed on ice for anesthetization about 10 min, hemolymph was subsequently extracted from the base of the chelate legs by using a sterilized syringe preloaded with ice-cold anticoagulant buffer (0.45 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM ethylenediaminetetraacetic acid, pH 4.6) [31]. Then hemocyte pellet was collected for RNA extraction after centrifugation at 800 × *g* for 15 min at 4 °C. Besides, heart, gill, hepatopancreas, stomach, intestine, connective tissue, muscle, and eye stalk of mud crabs were dissected, rinsed with sterile PBS, and pooled from three individual crabs for total RNA isolation. Then total RNA was used to investigate the tissue distribution of *SpCrus6* gene. Total RNA in the gill at 0, 2, 6, 12, 24, 48, and 72 h after challenged with different pathogens was extracted for the temporal expression analysis of *SpCrus6*. Simultaneously, crabs from the control were detected as well [3].

2.3. Total RNA isolation and cDNA synthesis

Total RNA was extracted from hemocytes and other collected tissues of the crabs using RNAiso Plus kit (TaKaRa, China). DNase I (Promega, USA) was added to the extracted total RNA to eliminate DNA contamination before the cDNA was synthesized with a First-Strand cDNA Synthesis Kit (TaKaRa, China) according to the manufacturer's instructions [30].

2.4. cDNA cloning of SpCrus6

The cDNA sequence encoding the deduced *Sp*Crus6 molecule was obtained through high-throughput transcriptome sequencing with a RNA mixture which was extracted from the hemocyte, gill, and hepatopancreas of normal mud crabs. A pair of gene-specific primers (*Sp*Crus6-CF: 5′-AAC TCC AGG TTG CAT CGC TCT-3′ and *Sp*Crus6-CR: 5′-GGA AGA AAT GAC ATG GTA GAT-3′), were designed to clone the cDNA sequence of target gene. And PCR was performed according to the following parameters: 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. At last, the targeted fragment was purified and subcloned into pMD-19T vectors and sequenced in company (Sangon, China).

2.5. Bioinformatics analysis

Identification of *Sp*Crus6 molecule with other crustins from invertebrates was performed using the online Basic Local Alignment Search Tool Program (BLASTP) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The ClustalX 2.0 program (http://www.ebi.ac.uk/tools/clustalw2) and the GENEDOC software were used to carry out multiple alignments of nucleotide sequences and amino acid sequences. The amino acids sequence was translated by online software (http://web.expasy.org/translate/). The pI and molecular weight (MW) were calculated using online software (http://web.expasy.org/compute_pi/). Signal peptide sequence was predicted with SignalP software. A neighbor-joining phylogenetic tree was constructed with MEGA 7.0, and 1000 bootstraps were selected to evaluate the reliability.

2.6. Quantitative real-time PCR (qRT-PCR)

QRT-PCR was performed to detect the relative expression level of *SpCrus6* in a real-time thermal cycler Quantstudio 6 Flex (ABI, USA) in

accordance with a previous protocol. A pair of gene-specific primers for *SpCrus6* (*Sp*Crus6-RF: 5′-GTG CTT GTG GTG GTG ATG GA-3′ and *Sp*Crus6-RR: 5′-CTC GGG ACA GAT AGG ACG GT-3′) were synthesized to amplify a 189 bp DNA target gene fragment. Another pair of specific primers for 18S rRNA gene of mud crab (18S-RF: 5′-CAG ACA AAT CGC TCC ACC AAC-3′ and 18S-RR: 5′-GAC TCA ACA CGG GGA ACC TCA-3′) were also synthesized as the internal reference. The total volume for PCR reaction was 20 μL (10 μL of 2 × Premix Ex Taq, 2 μL of cDNA, and 4 μL of each primer). QRT-PCR was done according to following parameters: 95 °C for 10 min; 40 cycles at 95 °C for 10 s and 60 °C for 60 s; and an increase from 60 °C to 95 °C.

All treatments were repeated three times beginning from the original immunity challenge. The $2^{-\Delta CT}$ method was used to calculate the relative expression level of *SpCrus6* in different tissues and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of *SpCrus6* in the gill after different pathogens challenged. Unpaired *t*-test was used to analyze the significant differences [25].

2.7. Recombinant expression and purification of rSpCrus6

In order to examine the biological effects of *SpCrus6*, recombinant *Sp*Crus6 was expressed in *E. coli* as GST-tagged fusion protein using pGEX-4T-1 expression system. PCR fragment encoding the mature peptide of *Sp*Crus6 was amplified with gene-specific primers (*Sp*Crus6EF: 5′-TAC TCA GAA TTC GGG AGG CCT AGT CCT TCT C-3′ and *Sp*Crus6ER: 5′-TAC TCA CTC GAG TGG GTC AGC AAG CAG ACA G-3′). The purified PCR product was subsequently digested and cloned into corresponding pGEX-4T-1 expression vector. Then recombinant plasmid (pGEX-4T-1-*SpCrus6*) was transformed into *E. coli* Transetta (DE3) chemically competent cells. Recombinant protein expression was induced by the addition of isopropyl-β-d-thiogalactoside (IPTG) to an ultimate concentration of 0.5 mM at 37 °C for 6 h, and analyzed by 15% SDS-PAGE. The bacteria were pelleted through centrifugation and re-suspended in PBS containing 0.1% Triton X-100 for probe sonication lysis. The recombinant *Sp*Crus6 protein (r*Sp*Crus6) was purified using Glutathoine Sepharose 4B chromatography (Novagen). The purified r*Sp*Crus6 was subsequently used for further experiments.

2.8. Antimicrobial assay

Antimicrobial activity of the purified r*Sp*Crus6 was tested against Gram-positive bacteria (*S. aureus*, *B. subtilis* and *Bacillus megaterium*), Gram-negative bacteria (*V. parahemolyticus*, *Vibrio alginolyticus*, *Vibrio harveyi* and *E. coli*), and fungi (*Pichia pastoris* and *Candida albicans*) by liquid growth inhibition assays in 96-well microtiter plates, respectively. In brief, serial of 2-fold dilutions of r*Sp*Crus6 or GST ranging from 0 to 50 μM were made in TBS (50 mM Tris-HCl and 150 mM NaCl; pH 7.5) in a volume of 100 μL on a 96-well microtiter plate, and each well was inoculated with 100 μL of a suspension of mid-log bacteria (10^5 CFU/mL) in Poor Broth (1% tryptone, 0.5% NaCl (w/v), pH 7.5). GST was used as negative control. Cultures were grown for 24 h at 37 °C or 28 °C (for *Vibrio* growth). The growth of bacteria was evaluated by measuring the culture absorbance at 600 nm using a microplate reader. The minimal growth inhibition concentration (MIC) in this study was defined as the lowest protein concentration harvesting significant growth inhibition compared with the negative control. All the values were averaged using three independent measurements [32].

2.9. Microorganism-binding assay

Based on the antimicrobial activity against bacteria and fungi in varying degrees results, western blot experiment was performed to investigate the antimicrobial mechanism. Briefly, the microorganisms (1×10^8 CFU) were incubated in 200 μL of 200 μg/mL r*Sp*Crus6 by gentle rotation for 30 min at room temperature. The microorganisms were subsequently collected, rinsed three times with 1 mL of TBS, and eluted with 200 μL of 7% SDS solution by mild agitation for 10 min. The microorganism pellets were washed three times with 1 mL of TBS. After the final centrifugation (10000 rpm for 5 min), the supernatant was sampled as elution and subjected to 15% SDS-PAGE. The bacterial pellets were also subjected to the SDS-PAGE after resuspension with TBS. And r*Sp*Crus6 was sampled to the gel as the positive control. Finally, proteins were transferred onto a nitrocellulose membrane, which was blocked with 5% skim milk in TBS and incubated with peroxidase-conjugated mouse monoclonal antibody against GST-tag. An ECL Western blot reagent kit was used to visualize r*Sp*Crus6. Whole experiments were performed individually in triplicate [3].

2.10. Microbial cell wall polysaccharides binding assay

To further study the action mechanisms of r*Sp*Crus6, microbial cell wall polysaccharides binding assay was performed by enzyme-linked immunosorbent assay (ELISA). In brief, 80 μL microbial cell wall polysaccharides (10 μg/mL), including LPS from *E. coli* 0111: B4, LTA from *S. aureus*, PGN from *S. aureus* and *B. subtilis*, and *β*-glucan from *L. digitata*, were respectively coated to the wells of a flat-bottom microtiter plate and incubated at 37 °C overnight. As a negative control, wells were incubated with 80 μL of 50 mM Tris-HCl. Each well was blocked with BSA (2 mg/mL, 200 μL) at 37 °C for 2 h. The solution was removed and rinsed with TBST (0.05% Tween 20 in TBS) four times. Subsequently, a series of diluted r*Sp*Crus6 or GST protein (0–6 μM in TBS containing 0.1 mg/mL BSA) was added to each well and incubated at 37 °C for 3 h. After washing with TBST four times and incubated with peroxidase-conjugated mouse monoclonal anti-GST antibody (1: 5000 diluted in TBS with 0.1 mg/mL BSA) at 37 °C for 2 h. After extensive rewashing the plate with TBST four times, it was developed with 0.01% 3,3′,5,5′-tetramethylbenzidine (Sigma). The reaction was stopped with 2 M H_2SO_4, and absorbance was tested at 450 nm wavelength. All experiments were repeated in triplicate [33].

2.11. Agglutinating assay

To provide a deeper understanding for the function mechanism of r*Sp*Crus6, nine microorganisms (used in microorganism-binding assay) were used again in the agglutination assay. Briefly, microorganisms at mid-logarithmic phase were obtained by centrifugation at 5000 × *g* for 5 min, washed with TBS four times, and resuspended in TBS to 2×10^8 cells mL^{-1} (for bacteria) or 2×10^7 cells mL^{-1} (for fungi). The microorganism suspension (30 μL) was incubated with a series of dilutions of r*Sp*Crus6 or GST in TBS (30 μL) at the protein concentration range of 0.4–6.25 μM in the absence or presence of 10 mM $CaCl_2$ at 28 °C for 1 h. GST was used as the negative control. The minimal agglutinating concentration (MAC) in this study was determined as the lowest protein concentration harvesting apparent agglutination function, compared with the negative control. Agglutination effect was observed by a light microscope [34]. All treatments were performed in triplicate.

2.12. GST pull-down assay for proteins interaction

Pull-down assay was performed according to the method described by Li et al. (2013), with slight modifications [35]. Recombinant *Sp*Crus6 and two major structural proteins of WSSV (VP28 and VP26), which were expressed in *E. coli* as His-tagged fusion proteins using pET-30-a expression system in our previous study, were used in this assay. A total of 150 μL glutathione-Sepharose 4B resin (50% beads slurry) after wash with PBS was incubated with a mixture of r*Sp*Crus6 (15 μg) and a viral protein (15 μg, rVP28 or rVP26) for 2 h at 4 °C. In the control group, r*Sp*Crus6 was replaced by GST tag protein. After incubation, the samples were washed thoroughly with PBS, and then proteins were eluted by adding PBS containing 10 mM reduced glutathione. The samples, including r*Sp*Crus6 (or GST tag protein), viral proteins, the

final wash, and elution were subjected to a 12.5% SDS-PAGE. And the results were analyzed after the gel was stained with Coomassie blue.

2.13. WSSV proliferation patterns in mud crab after pre-incubation with rSpCrus6

Thirty-six healthy mud crabs (about 100 g in weight) which were divided randomly into two groups were used for WSSV infection experiment. The purified WSSV (about 10^5 virion per crab) was pre-incubated with r*Sp*Crus6 (200 μg/mL) and GST tag protein (200 μg/mL) at 4 °C for 15 min and then injected into mud crabs of experiment group and control group, respectively. The gill from three mud crabs were harvested at 0, 24, 36, 48, 72, and 96 h post injection, and then were used to extract genomic DNA for the quantification of WSSV at different time-points. The WSSV copies in the gill were quantified according to a previously developed method [36]. Briefly, a standard plasmid containing a 619 bp DNA fragment of WSSV was constructed. Based on this DNA fragment, a pair of primers was designed to generate a shorter fragment as amplicon in the following SYBR green real-time PCR reaction. Subsequently, a series of ten-fold diluted plasmids (10^2 to 10^7 copies/μL) were used to generate a standard curve against their individual corresponding CT (Cycle Threshold) values. The WSSV copies of each sample were calculated automatically through the CT values generated by the real-time PCR machine (QuantStudio™6 Flex) based on the standard curve.

3. Results

3.1. Cloning of the SpCrus6 cDNA

By high-throughput transcriptome sequencing in our previous research, a cDNA sequence encoding the deduced *Sp*Crus6 molecule was obtained. The results of BLAST showed that this fragment was homologous to crustin molecules. According to the order of discovery in our research, this target gene was nominated as *SpCrus6* gene. After PCR amplification and gene sequence assembly, an 816 bp fragment was obtained (GenBank Accession No. MF431585). In brief, the ORF of *Sp*Crus6 encoded a protein of 113 amino acids, with a signal peptide sequence (SPS) consisting of 20 amino acids (Fig. 1). A WAP domain which was composed of 52-amino acid residues existed at the C-terminus. There were 8 conserved cysteines in the WAP domain, forming four disulfide bonds. And there were 5 proline residues, 5 glycine and 4 cysteine residues between SPS and WAP domain in *Sp*Crus6.

3.2. Multiple alignments and phylogenetic analysis

To investigate the amino acids sequence difference of *Sp*Crus6 with other crustin molecules, some invertebrates' crustin peptides were chosen to carry out sequences multiple alignments. The *Eriocheir sinensis* Crustin 1 (EU183310.1), *Fenneropenaeus chinensis* Crustin (AY871268.1), *Farfantepenaeus paulensis* Crustin (EF182747.1), *Farfantepenaeus subtilis* Crustin (EF450744.1), *Litopenaeus schmitti* Crustin (EF182748.1), *Panulirus japonicus* Crustin 2 (FJ797418.1), *Panulirus japonicus* Crustin 4 (FJ797420.1), *Penaeus monodon* Crustin 5 (FJ380049.1), *Scylla serrata* Crustin (HQ638025.1), *Portunus pelagicus* Crustin (JQ965930.1), and *Scylla tranquebarica* Crustin (JQ753312.1) were chosen to carry out sequences multiple alignments. The results showed that the sequences similarity was 45.3% among above-mentioned Crustin molecules. Among these crustin proteins including *Sp*Crus6, signal peptide sequence which included about twenty amino acid residues existed at the N-terminus, and a WAP domain existed at the C-terminus which included eight conserved cysteine residues (Fig. 2). Besides, four conserved cysteines existed between signal peptide sequences and WAP domains in all above-mentioned crustins.

The results of phylogenetic analysis for *Sp*Crus6 molecule and other crustins showed that *Sp*Crus6 molecule located in the same branch of evolutionary tree with *S. tranquebarica* Crustin (JQ753312.1), *S. serrata* Crustin (HQ638025.1), and *P. pelagicus* Crustin (JQ965930.1) (Fig. 3). This phenomenon showed they had more close evolutionary relationship.

3.3. Expression profiles for SpCrus6 gene

To investigate the expression profiles of *SpCrus6* gene, total RNA was extracted from normal, bacteria- and virus-challenged crabs. The expression patterns of *SpCrus6* in different normal tissues are shown in Fig. 4A. For example, *SpCrus6* transcripts were expressed in gill and stomach at a relatively high level and in hemocytes, heart, hepatopancreas, intestine, connective tissue, muscle, and eye stalk at a relatively low level (Fig. 4A). Due to the role in innate immunity defending system and the difficulty of extracting samples, gill was chosen to perform time course expression profiles. In the gill of WSSV challenged crabs, the expression level of *SpCrus6* was obviously up-regulated from 6 h to 48 h post-viral challenge and up-regulated to the maximum level at 24 h (Fig. 4B). In the gill of *V. parahemolyticus* challenged crabs, the expression level of *SpCrus6* was obviously up-regulated from 2 h to 48 h post-bacterial challenge and up-regulated to the maximum level at 6 h

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1   AACTCCAGGTTGCATCGCTCTCCTCAGCTTTGTTCCAGCATGCTTCGCATTGTGACTGTCGCCGTCGTGGTGCTTGTGGTGGTGATGGAT
1                                                   M  L  R  I  V  T  V  A  V  V  V  L  V  V  V  M  D
91  GCTGAGGCTGGGAGGCCTAGTCCTTCTCCAAGCTGCAGGAGCTGGTGCAAAAGGCCTGGACACCCTGAAAAGAACGCTTTTTACTGCTGT
18   A  E  A  G  R  P  S  P  S  P  S  C  R  S  W  C  K  R  P  G  H  P  E  K  N  A  F  Y  C  C
181 GACTTCGGGATTGGTACTGTTGGGAAACCTTTCGCAACACACCCTGGCAAGTGTCCCCACCGTCCTATCTGTCCCGAGGGTCTGTATACC
48   D  F  G  I  G  T  V  G  K  P  F  A  T  H  P  G  K  C  P  H  R  P  I  C  P  E  G  L  Y  T
271 AGGGGACCTGCTCCCACGGTATGTGCTCACGACGGCCAGTGCTCCAAGCACGAGAAGTGCTGTGCTGATGCCTGCCTTGAACACCACACC
78   R  G  P  A  P  T  V  C  A  H  D  G  Q  C  S  K  H  E  K  C  C  A  D  A  C  L  E  H  H  T
361 TGTCTGCTTGCTGACCCATAGTGACCTGGCCCTCAAAACCTTCACGTCCTGTCCTTCTTAATCCGAGAAAGTAGAAGGAAGAGAGAAATC
108  C  L  L  A  D  P  *
451 TGAGAAATTAAGATGAATTTGTTATTGTTGTTTGCTCGTTTAATATTTTCTCATGTTTTGAGTTTTGAAAAAGCTCTTTTGATGTAACGA
541 ATGTTTTCCCTTCAAATTAAATCAATAATGGCTTTTAATAATAGTATTTTTTTGTCCCTCATGGTTGATAAGCGACATGATAAACACACA
631 TATTATTATATTATGAGTAAGGTAAGAACTTATCGGAGAATTCAAAGGCCATTAATTCCCACAAGTGTCAATTTGTCATCCATGTCCATG
721 AATTTCCATAACTTTCCCTCAAAGCTTTCCATTGAATATATGAATGATCTGATTACTGAATATGTTCTAGTCATCTACCATGTCATTTCT
811 TCCCAG
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Fig. 1. cDNA and deduced amino acids sequences of *SpCrus6* from *S. paramamosain*. The signal peptides are shown in red letters and the stop code is shown by an asterisk (*). The whey acidic protein (WAP) domain is underlined. The conserved cysteine residues are highlighted in bold and shaded in gray. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

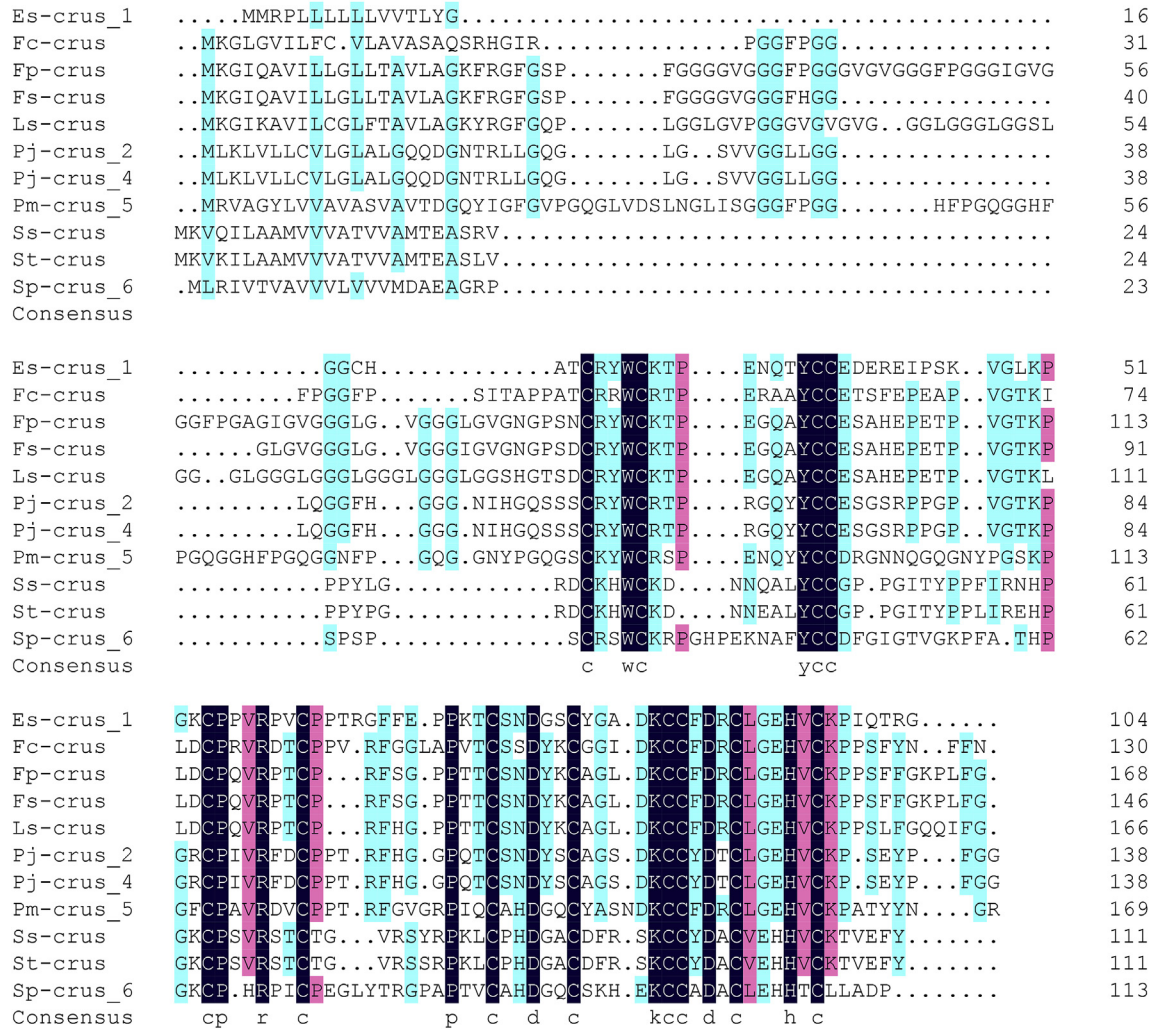

Fig. 2. Amino acids sequences alignment of *Sp*Crus6 with other crustins. The numbers on the right indicated the amino acid position of different sequences. Different colors represented the different conservations of amino acids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

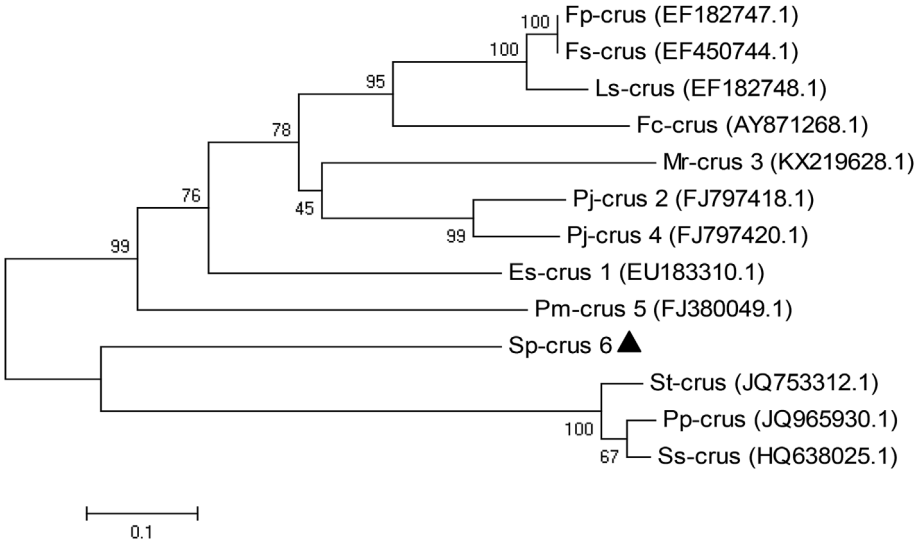

Fig. 3. Phylogenetic analysis of *Sp*Crus6 with other known crustins from invertebrates based on amino acids sequences. The neighbor-joining tree was constructed by molecular evolutionary genetics analysis (MEGA) software version 7.0. GenBank accession numbers followed the taxon names. *Sp*Crus6 was shown in black triangle.

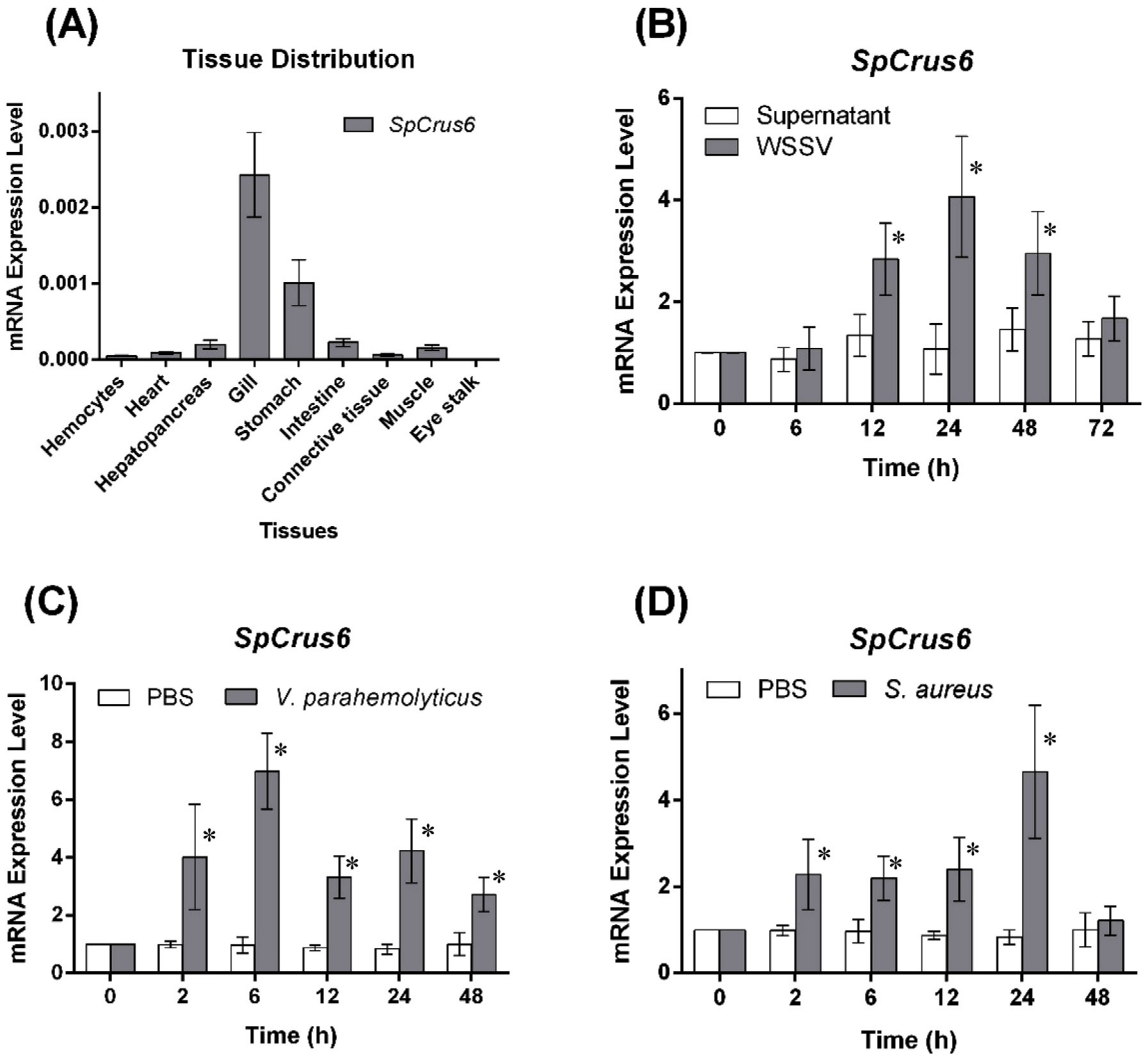

Fig. 4. Tissue distribution in normal crabs and time course expression patterns after challenged with different pathogens of *SpCrus6*. The transcripts of *SpCrus6* in normal tissues were examined by quantitative real-time PCR (A). Expression patterns of *SpCrus6* in gill after challenged with WSSV (B), *V. parahemolyticus* (C), and *S. aureus* (D) were shown in relative level to 18S rRNA inner control gene. Asterisks indicated the significant differences from the control (*: $P < 0.05$). Error bars represented ± standard deviation of 3 independent assays.

(Fig. 4C). In the gill of *S. aureus* challenged crabs, the expression level of *SpCrus6* was obviously up-regulated from 2 h to 24 h post-bacterial challenge and up-regulated to the maximum level at 24 h (Fig. 4D). All these changes showed that *SpCrus6* was associated with anti-virus and anti-bacteria responses.

3.4. Recombinant expression and purification

After IPTG inducing, r*Sp*Crus6 was expressed in the *E. coli* recombinant expression system. Theoretically predicted molecular mass of r*Sp*Crus6 was 39.4 kDa, which included the molecular mass of GST-tag. The SDS-PAGE results showed that the target band of r*Sp*Crus6 was consistent with the theoretical value (Fig. 5). Then r*Sp*Crus6 was purified with glutathione resin chromatography.

3.5. Antimicrobial activity of rSpCrus6

To test the antimicrobial activity of r*Sp*Crus6, liquid growth inhibition assays were performed. The minimal inhibitory concentrations against bacteria or fungi for r*Sp*Crus6 were listed in Table 1. Based on the MIC values, r*Sp*Crus6 showed superior inhibitory abilities against Gram-positive bacteria (*B. megaterium*, *S. aureus*, and *B. subtilis*). The MIC values were 0.8, 1.6, and 3.12 μM, respectively. The MIC values for four Gram-negative bacteria (*V. parahemolyticus*, *V. alginolyticus*, *V. harveyi* and *E. coli*) were 25 μM. And the MIC values for two fungi (*P. pastoris* and *C. albicans*) were respectively 25 and 6.25 μM (Table 1).

3.6. Microorganism-binding activity of rSpCrus6

To further study the antibacterial mechanism of r*Sp*Crus6, microorganism-binding assay was carried out. The results showed that r*Sp*Crus6 could strongly bind to two Gram-positive bacteria (*B. subtilis* and *B. megaterium*) and three Gram-negative bacteria (*V. alginolyticus*, *V. parahemolyticus*, and *V. harveyi*) (Fig. 6). And r*Sp*Crus6 could weakly bind to *S. aureus* and two fungi (*P. pastoris* and *C. albicans*). These results indicated that *Sp*Crus6 might be involved in binding activities to bacterial cells.

3.7. Microbial cell wall polysaccharides binding activity of rSpCrus6

To deep investigate r*Sp*Crus6's binding activity to bacterial cell wall components, ELISA was performed. The results showed that the binding abilities increased progressively with the increase of proteins concentrations (Fig. 7). And r*Sp*Crus6 possessed superior binding activities

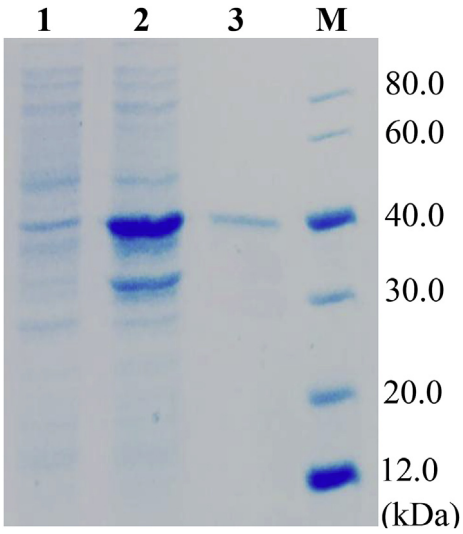

Fig. 5. SDS-PAGE analysis of recombinant *Sp*Crus6 expressed with a GST-tag in *E. coli*. Lane M, protein marker; lane 1, total protein obtained from *E. coli* without induction; lane 2, total protein obtained from *E. coli* with IPTG induction; lane 3, recombinant *Sp*Crus6 purified with glutathione resin.

Table 1
Minimal inhibitory concentrations of r*Sp*Crus6. Minimal inhibitory concentration is defined as the lowest protein concentration harvesting visible growth inhibition function, compared with the negative control.

Microorganism	Minimal inhibitory concentrations (μM)
Gram$^+$	
S. aureus	< 1.6
B. megaterium	< 0.8
B. subtilis	< 3.12
Gram$^-$	
V. alginolyticus	> 25
V. parahemolyticus	> 25
V. harveyi	> 25
E. coli	> 25
Fungi	
P. pastoris	> 25
C. albicans	< 6.25

to LPS (from *E. coli* 0111: B4), LTA (from *S. aureus*), PGN (from *S. aureus* and *B. subtilis*) and *β*-glucan (from *L. digitata*).

3.8. Agglutinating activities of rSpCrus6

On the base of microorganism-binding activities and microbial cell wall polysaccharides binding activities, agglutinating assays were carried out. Nine microorganisms which were used in the microorganism-binding assay were used again in this experiment. The results of agglutinating assays showed that r*Sp*Crus6 showed strong agglutinating activities to three Gram-positive bacteria (*S. aureus*, *B. subtilis* and *B. megaterium*). According to the MAC values in Table 2, we also found Ca^{2+} could increase the agglutinating ability of r*Sp*Crus6. The agglutinating activities to Gram-negative bacteria and fungi were not found (Table 2). Besides, *B. megaterium* was chosen as a representative to illustrate intuitively the agglutination activities of r*Sp*Crus6 (Fig. 8).

3.9. Interactions between rSpCrus6 and WSSV structural proteins

To investigate the likely antiviral mechanism of *Sp*Crus6 *in vivo*, GST pull-down assay was carried out to test the binding ability of r*Sp*Crus6 to the specific structural proteins of WSSV. And VP26 and VP28, as the candidate binding proteins of WSSV, were examined in this study. The results showed that r*Sp*Cru6 could bind to rVP26 but not to rVP28, whereas GST protein could not interact with viral protein rVP26 or rVP28 (Fig. 9). These results indicated that r*Sp*Crus6 could specifically interact with rVP26.

3.10. rSpCrus6 suppressed reproduction of WSSV

To confirm that whether *Sp*Crus6 can inhibit the proliferation of WSSV *in vivo*, the proliferation patterns of r*Sp*Crus6-treated group (experiment group) and GST tag protein-treated group (control group) in mud crabs were compared. As shown in Fig. 10, the load of virion in crabs of control group began to accelerate at 36 h post-injection, extending to the end of this experiment. By contrast, in the experiment group, the number of WSSV began to increase obviously at 48 h post-injection. Furthermore, the loads of WSSV in experiment group at 48, 72, and 96 h were much lower than those in the control group (Fig. 10). These results indicated that r*Sp*Crus6 could suppress the reproduction of WSSV to a certain degree.

4. Discussion

Crustins are a kind of cationic AMPs, which contain a WAP domain at the molecule C-terminus. According to the sequence difference between SPS and WAP domain, crustins were divided initially into three sub-families [22]. Type-I crustins contain a cysteine-rich region between SPS and WAP domain, which usually reported in crayfish, lobster, and crab [37]. Type-II crustins contain a glycine-rich and a cysteine-rich region between SPS and WAP domain, and they are usually found in shrimp. Type-III crustins have a proline-arginine region between SPS and WAP domain [22]. With the discovery of more AMPs, classification has been updated in recent years. There are two new additional subfamilies, including type-IV and type-V crustins [21]. Type-IV crustins contain two WAP domains, and type-V crustins which are similar to type-I crustins contain an extra aromatic amino acid-rich region between the cysteine-rich region and the WAP domain [38]. In present study, we identified a new crustin gene from *S. paramamosain*, which was named as *SpCrus6* gene. *SpCrus6* gene was 816 bp long. The

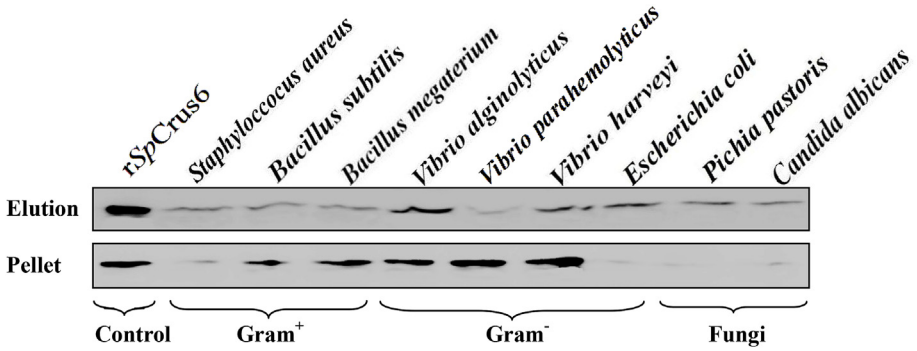

Fig. 6. r*Sp*Crus6 was detected by Western blot assay after treatment with bacteria (Gram$^+$ and Gram$^-$) and fungi. r*Sp*Crus6 was taken as positive control. Up panel, elution fractions; bottom panel, final pellet fractions. Western blot experiments were independently conducted and repeated three times. And peroxidase-conjugated mouse monoclonal antibody against GST-tag was used.

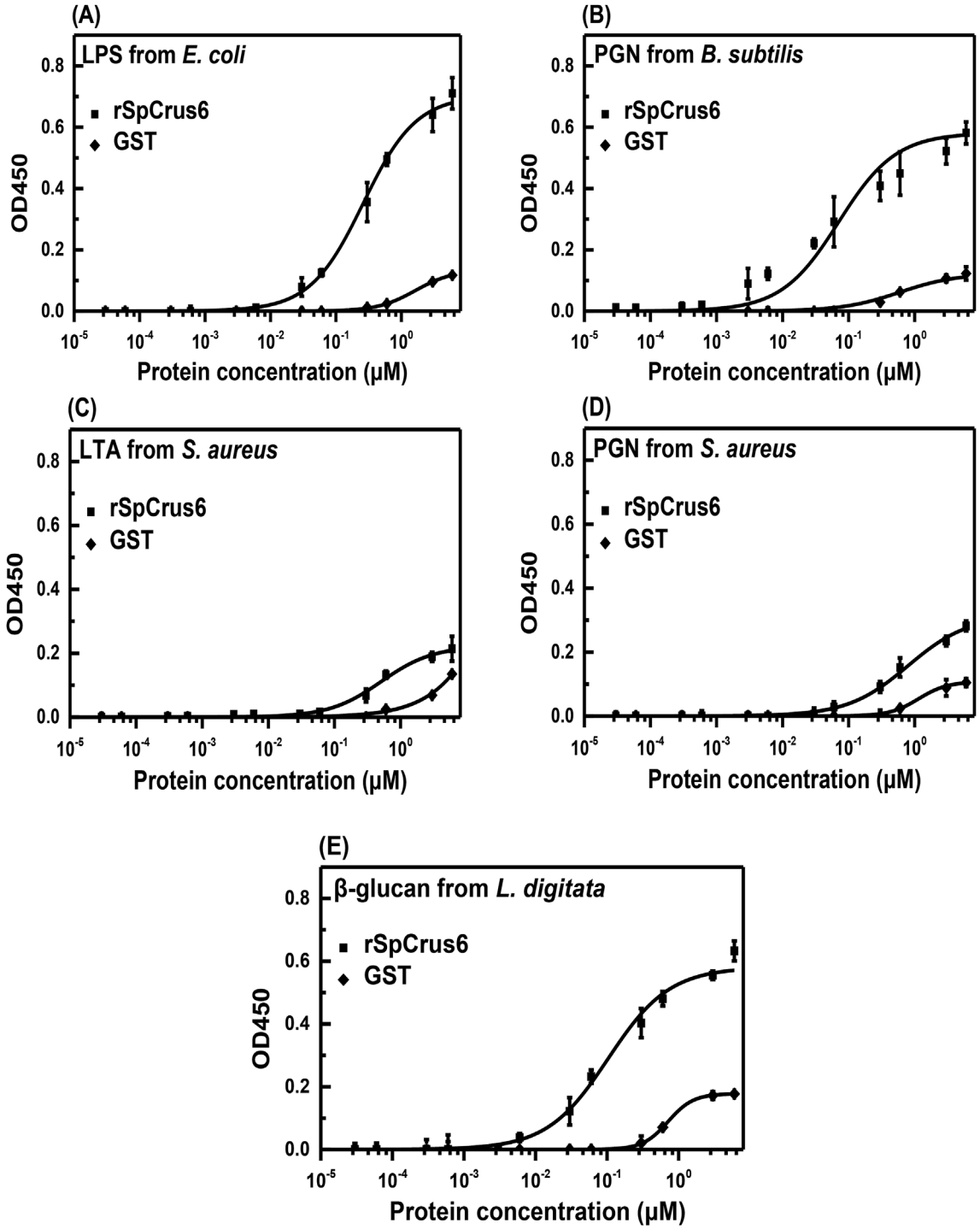

Fig. 7. ELISA analysis of binding affinity to microbial cell wall polysaccharides of r*Sp*Crus6. GST was used as negative control. The results were represented as mean values of three independent experiments with standard deviation.

ORF contained 113 amino acids, with a SPS consisting of 20 amino acids at the N-terminus (Fig. 1).

There was a WAP domain including 52 amino acid residues at the C-terminus of *Sp*Crus6 molecule. Eight conserved cysteines existed in the WAP domain, which could form four disulfide bonds. And there were 5 proline, 5 glycine and 4 cysteine residues between SPS and WAP domain in *Sp*Crus6. According to the latest classification principle, *Sp*Crus6 belonged to Type-I crustins, but there were also 5 proline residues between SPS and WAP domain (Fig. 1). These features indicated *Sp*Crus6 was a new type of crustin molecule. Based on the multiple alignment results, SPS, cysteine-rich region and WAP domain were found in all chosen crustin proteins (Fig. 2). The results of phylogenetic analysis for *Sp*Crus6 molecule and other crustins showed that *Sp*Crus6 molecule located in the same branch of evolutionary tree with *S. tranquebarica* Crustin (JQ753312.1), *S. serrata* Crustin (HQ638025.1), and *P. pelagicus* Crustin (JQ965930.1) (Fig. 3). This result also verified that *S. paramamosain* had relatively close evolutionary relationships with *S. tranquebarica*, *S. serrata*, and *P. pelagicus*.

To understand *SpCrus6* gene's roles *in vivo*, tissues distribution and time-course expression profiles after pathogens infection were studied. *SpCrus6* mRNA transcript mostly distributed in gill and stomach. And it was expressed at a relatively low level in hemocytes, heart,

Table 2
Agglutinating activity of r*Sp*Crus6. Minimal agglutinating concentration is defined as the lowest protein concentration obtained obvious agglutination, compared with the negative control. '−' means no obvious agglutination was observed with protein concentration of 6.25 μM.

Microorganisms	Minimal agglutinating concentrations (μM)	
	r*Sp*Crus6 + Ca^{2+}	r*Sp*Crus6
Gram$^+$		
S. aureus	< 0.8	< 6.25
B. subtilis	< 3.12	< 6.25
B. megaterium	< 0.4	< 0.4
Gram$^-$		
V. alginolyticus	−	−
V. parahemolyticus	−	−
V. harveyi	−	−
E. coli	−	−
Fungi		
P. pastoris	−	−
C. albican	−	−

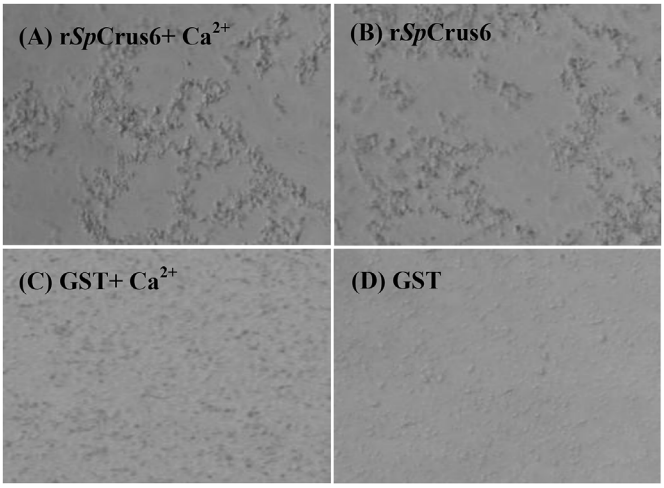

Fig. 8. Based on the MAC values, *B. megaterium* was chosen as a representative to illustrate the agglutination activities of r*Sp*Crus6 to microorganisms. GST was used as negative control.

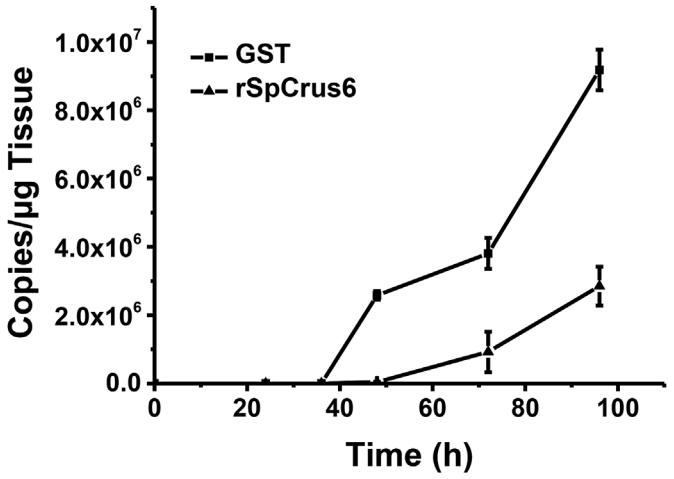

Fig. 10. WSSV proliferation patterns in mud crab after pre-incubation with r*Sp*Crus6 or GST proteins. This figure showed the number of WSSV (Copies/μg Tissue) in gill at different time-points.

hepatopancreas, intestine, connective tissue, muscle, and eye stalk (Fig. 4A). In the gill of WSSV challenged crabs, *SpCrus6* mRNA transcript was obviously up-regulated after viral infection and reached the maximum at 24 h post infection (Fig. 4B). In the gill of *V. parahemolyticus* challenged crabs, *SpCrus6* mRNA transcript was also obviously up-regulated and reached the maximum at 6 h post infection (Fig. 4C). In the gill of *S. aureus* challenged crabs, *SpCrus6* mRNA transcript was obviously up-regulated to the maximum level at 24 h post infection (Fig. 4D). At the same time, the differences in the changes of *SpCrus6* gene expression levels were significant. These results about gene expression profiles were consistent with the patterns of most crustin proteins reported in crustaceans [3,7,25,37,39–41]. All these results showed that *SpCrus6* mRNA could be mobilized quickly to defend against pathogens infection, including bacteria and virus. The gene expression levels' obvious changes demonstrated that *SpCrus6* was associated with antimicrobial and antiviral responses.

On the base of the recombinant expression and purification, liquid growth inhibition assays were performed. After comparing with other crustins' biological activities, we found most crustins possessed antimicrobial activities against Gram-positive bacteria [26,39,42–44]. Meanwhile, some crustins were reported that they could inhibit Gram-

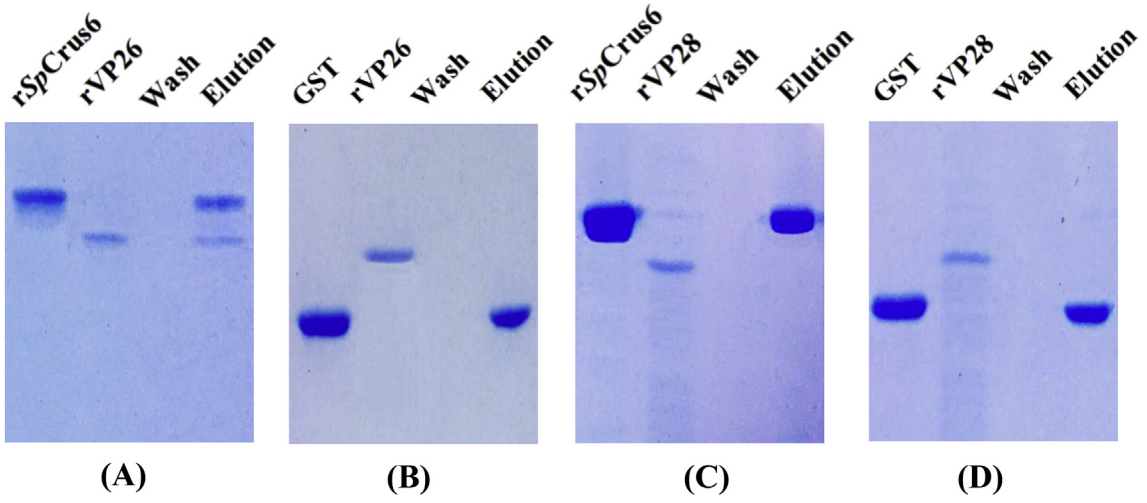

Fig. 9. GST Pull-down assays were conducted to test the interactions between r*Sp*Crus6 and viral proteins. (A) r*Sp*Crus6 and rVP26 were mixed with glutathione-Sepharose 4B resin. r*Sp*Crus6 and rVP26 were eluted simultaneously by elution buffer but not with wash buffer. (B) GST and rVP26 were mixed with glutathione-Sepharose 4B resin. Only GST was eluted by elution buffer. (C) r*Sp*Crus6 and rVP28 were mixed with glutathione-Sepharose 4B resin. r*Sp*Crus6 was eluted by elution buffer but not with wash buffer. (D) GST and rVP28 were mixed with glutathione-Sepharose 4B resin. Only GST was eluted by elution buffer.

negative bacteria growth [45–47]. In this study, the liquid growth inhibition assays' results showed that r*Sp*Crus6 possessed superior inhibitory abilities against Gram-positive bacteria (*B. megaterium*, *S. aureus*, and *B. subtilis*). And r*Sp*Crus6's inhibitory abilities against four Gram-negative bacteria (*V. parahemolyticus*, *V. alginolyticus*, *V. harveyi* and *E. coli*) and two fungi (*P. pastoris* and *C. albicans*) were not very strong enough (Table 1). Microorganism-binding assay's results showed that r*Sp*Crus6 could strongly bind to two Gram-positive bacteria (*B. subtilis* and *B. megaterium*) and three Gram-negative bacteria (*V. alginolyticus*, *V. parahemolyticus*, and *V. harveyi*) (Fig. 6). And r*Sp*Crus6 could weakly bind to *S. aureus* and two fungi (*P. pastoris* and *C. albicans*). After comprehensively analyzing the results of the liquid growth inhibition assays and the microorganism-binding assay, some confusion was found. For example, r*Sp*Crus6 possessed more potent inhibitory ability against Gram-positive bacteria than Gram-negative bacteria. However, the results of the microorganism-binding assay showed that r*Sp*Crus6 could strongly bind to three Gram-negative bacteria, which binding activity was similar to that of some Gram-positive bacteria. These results implied that though microorganism-binding activities were required for the inhibitory abilities, the result that r*Sp*Crus6 had the stronger microorganism-binding activities did not mean that it had the potent inhibitory abilities.

In order to have a better understanding for the binding mechanisms between r*Sp*Crus6 and microorganisms and reveal the underlying antimicrobial mechanism of *Sp*Crus6, microbial cell wall polysaccharides binding assays were conducted. The results showed that the binding abilities increased gradually with the increase of r*Sp*Crus6 concentrations (Fig. 7). And r*Sp*Crus6 had superior binding activities to LPS, LTA, PGN and *β*-glucan. This indicated that the microorganism-binding activities of r*Sp*Crus6 might largely rely on its binding activities to these four different kinds of polysaccharides. As for the likely antimicrobial mechanism, according to the previous reports, although AMPs have been considered as membrane-active agents disrupting the phospholipid bilayers of bacteria [48], several types of AMPs did not directly alter membrane integrity but target some specific components on the bacterial surface to exhibit antimicrobial activities [49,50]. Many microbial polysaccharides were targets of several types of AMPs, such as ALFs and crustins [27–29,50–53] Thus, we speculated that the antimicrobial activities that r*Sp*Crus6 possessed may also be via the interaction with the specific components of bacterial cell wall.

Besides, agglutinating assays were performed in present study. Previous reports showed that bacterial agglutination was a major mode of crustins in exerting their antimicrobial activities [54]. In their theory, some crustins displayed considerable bacterial agglutination activity by crosslinking the surface components of bacterial cells and forming lattices. In this study, r*Sp*Crus6 could well agglutinate three Gram-positive bacteria (*S. aureus*, *B. subtilis* and *B. megaterium*). And Ca^{2+} could increase the agglutinating ability of r*Sp*Crus6, to some extent (Table 2). The agglutinating activities to Gram-negative bacteria and fungi were not found. These results suggested that agglutination was another manner by which r*Sp*Crus6 exerted its antimicrobial activity against Gram-positive bacteria.

On the base of the expression profiles of *SpCrus6* gene's in the WSSV-challenged gill of *S. paramamosain*, proteins interactions and WSSV proliferation patterns were tested to study the antiviral functions. There were at least 59 structural proteins including 35 envelope proteins and 9 nucleocapsid proteins in WSSV [55]. These envelope proteins played important roles in binding the host cells surface receptors and infecting the host cells. Especially, the envelope protein VP28 located on the surface of the virus particle, which played a crucial role in the initial steps of the systemic WSSV infection process in shrimp [56]. And VP26 was a tegument protein, which located between the virus envelope and nucleocapsid. It contained an actin-binding motif in the molecular structure. VP26 was considered to be an important linker protein between the envelope and envelope proteins, or the envelope and nucleocapsid proteins of virions [57]. Likewise, VP26 was proved to facilitate the attachment of the virions to the shrimp cells [58]. More evidences showed that VP26 and VP28 played a major role in infection process, especially the interaction with the host cells. Thus, VP26 and VP28 were chosen to test the possible interactions between r*Sp*Crus6 and viral proteins of WSSV. The results of GST pull-down assay showed that r*Sp*Crus6 could specifically interact with rVP26 but not with rVP28. We speculated r*Sp*Crus6 displayed antiviral activity by initially recognizing and binding to VP26 of WSSV. This is because the links between the envelope and envelope proteins, or the envelope and nucleocapsid proteins of WSSV virions may be blocked in the presence of r*Sp*Crus6, which will affect the assembly and proliferation of WSSV. After detecting the WSSV proliferation patterns of r*Sp*Crus6-treated group and GST tag protein-treated group in mud crabs, we found that r*Sp*Crus6 could obviously suppress the reproduction of WSSV to a certain degree, in the whole testing process. Taken together, *Sp*Crus6 was a multifunctional immune effector, which played an important role in defending against pathogens' infection in *S. paramamosain*, including bacteria and virus.

Acknowledgements

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