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Two novel serine proteases from *Scylla paramamosain* involved in the synthesis of anti-lipopolysaccharide factors and activation of prophenoloxidase system

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ABSTRACT

Serine proteases (SPs) are important in various immune responses, including prophenoloxidase (proPO) activation, antimicrobial peptides (AMPs) synthesis, and hemolymph coagulation in invertebrates. In this study, SP3 and SP5 of mud crab (*Scylla paramamosain*) were studied. SP3 and SP5 were expressed in all examined tissues (mainly in hemocytes), and are associated with the immune responses of mud crab to *Vibrio parahemolyticus* and *Staphylococcus aureus*, as well as interacted with TRAF6, and are involved in the activation of anti-lipopolysaccharide factors (ALFs) probably through the TLR/NF-κB pathway. Depletion of SP3 inhibited the expression of ALF1, ALF2, ALF3, and ALF6, while knockdown of SP5 significantly decreased ALF5, and ALF6. Furthermore, both SP5 and TRAF6 regulated the PO activity in the hemolymph of mud crab. Overexpression assay showed that both SP3 and SP5 could enhance the promoter activities of ALFs in mud crab. Taken together, the results of this study indicate that SP3 and SP5 might play important roles in the immune system of mud crab against pathogen invasion.

1. Introduction

Multicellular organisms possess both innate and adaptive immunity for defense against invasion by foreign substances [1]. Innate immunity is essential for removing pathogens in the early stages of an immune response [2]. Invertebrates lack immunoglobulins and have developed unique forms of immune response to detect and recognize substances (i.e., lipopolysaccharides (LPS), peptidoglycan (PGN), and β-1, 3-glucans), which are present on the surface of microbes [3].

Melanization is a rapid and effective innate immune response mechanism to pathogen infection, which is controlled by the prophenoloxidase (proPO) system in invertebrates [4]. The proPO activating system is initially triggered by pattern-recognition proteins (PRPs) that recognize molecular patterns such as LPS, PGN, and β-1,3-glucans, found on microbial pathogens. There is then the initiation of a serine proteinase cascade that leads to the conversion of zymogenic proPO into catalytically active phenoloxidase, which then oxidizes phenolic substrates, resulting in melanin formation at the site of injury or around invading pathogens [5,6].

The TLR-MyD88-Tube/Pelle-TRAF6-NF-κB signaling pathway is evolutionarily conserved in antibacterial and antiviral defense in *Drosophila* and humans [7,8]. The Toll signaling pathway of invertebrates (for example, *Bombyx mori, Aedes aegypti* and *Drosophila*) cannot recognize pathogen-associated molecular patterns directly as in mammals [9–11]. However, an extracellular serine protease (SP) can cleave Spätzle (a cysteine knot cytokine), enabling it to bind to Toll and induce the synthesis of antimicrobial peptides (AMPs) [9].

Almost one-third of all known proteases are SPs, which are the largest class of proteases widely found in viruses, bacteria and eukaryotes [12,13]. SPs are characterized by a conserved C-terminal catalytic serine protease domain, with residues of the catalytic triad Histidine- H, Aspartic acid- D and Serine- S [14,15]. Serine protease homologs (SPHs) lack amidase activity due to the substitution of one or more key residues within the catalytic triad, but they can be cofactors required for proPO activation and negatively regulate the melanization response [16–18]. SPs can be structurally divided into non-clip domain SPs and clip domain SPs (cSPs), containing one or more clip domain(s) at the *N*-terminus [19]. Clip domains consist of 35–55 residues and are

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Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	PCR objective
Oligo-	AAGCAGTGGTATCAACGCAGAGTACXXXXX	First-Strand cDNA synthesis
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	RACE-PCR
UPM (short)	CTAATACGACTCACTATAGGGC	RACE-PCR
NUP	AAGCAGTGGTATCAACGCAGAGT	RACE-PCR
SP3-GSP3-1	CTCTGTGCAAGTCATGAACCAAGAGACATCC	3′RACE
SP3-GSP3-2	TTGTGACTCAAGACGCCCGAGGACG	3′RACE
SP3-GSP5-1	TGGCCGGGAAATATAATGGCACCGACCTCT	5′RACE
SP3-GSP5-2	GACCTGGAACATCTGCCTGTCCTGCCG	5′RACE
SP5-GSP3-1	TGCTGCTCGCTCTGGTTTGG	3′RACE
SP5-GSP3-2	CGATATTGCGCTGATAAGGCTCGATAAGGA	3′RACE
SP5-GSP5-1	GACCAGTAACCTTGACGCCCCTTCCG	5′RACE
SP5-GSP5-2	ACATCCACTCTATCGCAGTTCAGCTATTCCG	5′RACE
M13F	CGCCAGGGTTTTCCCAGTCACGAC	PCR screening
M13R	AGCGGATAACAATTTCACACAGGA	PCR screening
Q-SP3F	CGTGGAATACCAACCTCCTACCTG	qRT-PCR
Q-SP3R	AGGGTCTCCAGCACACACAGTCTC	qRT-PCR
Q-SP5F	CTCCAGTGCATCCCCATCAGG	qRT-PCR
Q-SP5R	GGACTCCCGTCGTCAGCAAAAC	qRT-PCR
β-ActinF	GCGGCAGTGGTCATCTCCT	qRT-PCR
β-ActinR	GCCCTTCCTCACGCTATCCT	qRT-PCR
siSP3F	GGTTATGTATCCTGGGTAT	RNAi
siSP3R	ATACCCAGGATACATAACC	RNAi
siSP5F	GCTGGTCAGACGAGGATAT	RNAi
siSP5R	ATATCCTCGTCTGACCAGC	RNAi
siTRAF6F	GCUUCUCCCAGCUUGCAAUTT	RNAi
siTRAF6R	AUUGCAAGCUGGGAGAAGCTT	RNAi
siGFPF	GGCUACGUCCAGGACCGCACC	RNAi
siGFPR	UGCGCUCCUGGACGUAGCCUU	RNAi
Q-siSP3F	GAGACTGTGTGTGCTGGAGACCC	qRT-PCR-RNAi
Q-siSP3R	CAGGCGAGGTAAGGCTTGTGG	qRT-PCR-RNAi
Q-siSP5F	GACCACGCAAGCACCGACAT	qRT-PCR-RNAi
Q-siSP5R	ACACCACCTGAGGCTGGAACC	qRT-PCR-RNAi
Q-siTRAF6F	CCAATTGACAACACCCCTCTG	qRT-PCR-RNAi
Q-siTRAF6R	GGCGGAACACTCATTCGGAC	qRT-PCR-RNAi
SpALF1F	AACTCATCACGGAGAATAACGC	qRT-PCR-RNAi
SpALF1R	CTTCCTCGTTGTTTTCACCCTC	qRT-PCR-RNAi
SpALF2F	TGTCGCTCAGGGACTCATCAC	qRT-PCR-RNAi
SpALF2R	GGAGATCACGGGAGAGTGAATG	qRT-PCR-RNAi
SpALF3F	GAACGGACTCATCACACAGCAG	qRT-PCR-RNAi
SpALF3R	CACTTCCTTGTTCTCTTCGCTC	qRT-PCR-RNAi
SpALF4F	CACTACTGTGTCCTGAGCCGC	qRT-PCR-RNAi
SpALF4R	GTCCTCGCCTTACAATCTTCTG	qRT-PCR-RNAi
SpALF5F	CTTGAAGGGACGAGGTGATGAG	qRT-PCR-RNAi
SpALF5R	TGACCAGCCCATTCGCTACAG	qRT-PCR-RNAi
SpALF6F	ACAGGGCTATCGCAGACTTCG	qRT-PCR-RNAi
SpALF6R	GCACCTCTTTGGCACACTATTTG	qRT-PCR-RNAi
SpproPOF	AGCGAACAGAAGCAAGTG	qRT-PCR-RNAi
SpproPOR	AGCGAACAGAAGCAAGTG	qRT-PCR-RNAi
Sp-cSPF	CTGGATGACACTACTCGGGAAG	qRT-PCR-RNAi
Sp-cSPR	CCTCATTGTCGTTGTAGTCGTC	qRT-PCR-RNAi
SpcSPF	CGAGGCAAGCCAAGCAGT	qRT-PCR-RNAi
SpcSPR	TCTCTCCGTCCACCGCAG	qRT-PCR-RNAi
SP4F	CAACTCCCGTGGTCAAGTGTCAG	qRT-PCR-RNAi
SP4R	TTCCATTCTCCGTGAAGCCCC	qRT-PCR-RNAi
E-SP3F	CCGGAATTCATTATATTTCCTGGCCAACTG	Recombinant expression
E-SP3R	CCGCTCGAGAGTGAACGCTATTTTTTTTATCC	Recombinant expression
E-SP5F	CCGGAATTCCAGTCCTCCCCAGATTGTAGCA	Recombinant expression
E-SP5R	CCGGAATTCCAGTCCTCCCCAGATTGTAGC	Recombinant expression
SP3-His-F	CCGGAATTCATGATTATATTTCCTGGCCAACTG	Luciferase Reporter and Co-IP
SP3-His-R	CCGCTCGAGAGTGAACGCTATTTTTTTTATCC	Luciferase Reporter and Co-IP
SP5-Flag-F	CCGGAATTCATGGATTACAAGGATGACGACGATAAGCAGTCCTCCCCAGATTGTAGCA	Luciferase Reporter and Co-IP
SP5-Flag-R	GGGGTACCTCACTCCAGAATCCAACTCCTGTAGGC	Luciferase Reporter and Co-IP

Note: Restriction enzyme sites are underlined; x = undisclosed base in the proprietary SMARTer oligo sequence.

strictly conserved by six cysteine residues, forming three pairs of disulfide bonds (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6, respectively) [18,20]. The cSPs were first discovered in the Japanese horseshoe crab (*Tachypleus tridentatus*), as a proclotting enzyme in the coagulation system [21]. Depending on the substrate specificity of SPs, they can also be subdivided into trypsin, chymotrypsin, elastase or collagenase [15].

SPs are known to participate in various physiological processes such as digestive and blood clotting, cellular and humoral immunity, embryonic development and tissue remodeling [22,23]. SPs are typically synthesized as zymogens, which are then activated by a specific and limited proteolytic cleavage at a specific peptide bond [24]. Activated SPs intend activate proPO or the Toll-ligand Spätzle in immune cascade pathways [11,25]. Activated SPs are regulated by serine protease inhibitors found in hemolymph [26,27]. It has previously been shown in

Pacific white shrimp (*Litopenaeus vannamei*) that alpha-2-macroglobulin (A2M) inhibits PO activity [28]. Caspase-1 is reported to regulate the proPO system in crayfish [29].

Tumor necrosis factor receptor-associated factor 6 (TRAF6) was initially identified as a signal transducer for interleukin-1 (IL-1) [30]. Among the components associated with the Toll/TLR signaling pathway, TRAF6 is known to be a crucial signal transducer conserved from *Drosophila* to human [31]. TRAF6 prevents the mitochondrial translocation of p53 and spontaneous apoptosis by promoting K63-linked ubiquitination of p53 in the cytosol [32]. In crustaceans, *Scylla paramamosain* TRAF6 (*Sp*-TRAF6) has been reported to be important in response to invasive pathogens (such as *Vibrio parahaemolyticus* and LPS) *via* regulation of anti-lipopolysaccharide factor (ALF) gene expression [33].

Our preliminary LC-ESI-MS/MS studies have shown that in mud crab (*S. paramamosain*), two novel SPs are able to interact with TRAF6 through protein-protein interaction (unpublished data). Therefore, in this study, the two novel SPs (SP3 and SP5) of mud crab were cloned and characterized so as to delineate the interplay between SPs and TRAF6. The technique of RNA interference (RNAi) was applied to the functions of SP3 and SP5 in the regulation of ALFs and proPO transcription in mud crab. Both SP5 and TRAF6 were shown to regulate the PO activity in the hemolymph of mud crab. The results from the present study further extends our understanding of the Toll signaling pathways and the proPO system in mud crab.

2. Materials and methods

2.1. Crabs tissue collection and immune challenges

A total of forty-eight *S. paramamosain* (body weight 80–100 g) were obtained from a farm in Niutianyang (Shantou, Guangdong, China), and acclimatized in laboratory tanks for one week before further processing. The salinity (8‰) and temperature (24–28 °C) were maintained during the acclimatization period. After the period of acclimatization, tissues (hemocytes, muscle, subcuticular epidermis, gills, hepatopancreas, midintestines, brain, and heart) were sampled from mud crabs, rinsed with 0.1% diethylpyrocarbonate (DEPC)-treated water and immediately dipped into liquid nitrogen for subsequent total RNA extraction using TRIzol® Reagent (Ambion, USA). For the challenge experiments, 200 μL of *V. parahaemolyticus* (1×10^7 *cfu* mL^{-1}) or 200 μL of *Staphylococcus aureus* (1×10^5 *cfu* mL^{-1}) were injected into the base of the fourth leg of each crab. For blank control, 200 μL of 0.8% NaCl (normal saline, NS) (Sangon Biotech, Shanghai, China) was used. The experiments were conducted under laboratory conditions (salinity: 8‰ and temperature: 24–28 °C). Hemolymph from three crabs per group was collected at 0, 6, 12, 24, 48, and 96 h post-injection (hpi) into sterilized tubes containing an equal volume of ice-cold acid citrate dextrose (ACD) anticoagulant buffer (1.32% sodium citrate, 0.48% citric acid, 1.47% glucose). Samples were immediately centrifuged at 800 × *g* for 20 min at 4 °C to separate the hemocytes, which was then used for RNA extraction.

2.2. cDNA cloning

Hemocytes RNAs were incubated for 30 min at 37 °C with 10 units of DNase I (Takara, Dalian, China) to remove trace genomic DNA contamination. The quality and quantity of purified RNA were determined by measuring the ratio of 260/280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), and the RNA integrity confirmed through analysis on a 1.5% (w/v) agarose gel. A total of 5 μg RNA was reverse transcribed using M-MLV First-Strand cDNA Synthesis Kit (Invitrogen, USA).

Partial cDNA sequences of SPs were obtained from our previous high-throughput transcriptomic data. The complete cDNA of SPs was amplified through 3′RACE and 5′RACE PCR with the SMARTer™ RACE cDNA Amplification Kit (Clontech, USA), using touch-down PCR and nested PCR strategy with gene specific primers (Table 1). After identification by agarose gel electrophoresis, the expected DNA fragment was excised and purified using the SanPrep Column DNA Gel Extraction Kit (Sangon, Shanghai, China). Purified DNA fragments were subcloned into a pMD®19-T vector (Takara, Dalian, China) and then transformed into *Escherichia coli*. Positive recombinant clones were identified by PCR screening with M13R and M13F primers and sequenced by a commercial company (BGI, Shenzhen, China).

2.3. Bioinformatics analysis

The BLAST program (http://www.ncbi.nlm.nih.gov/blast/) was used to analyze the nucleotide sequences. The deduced amino acid sequence was obtained with the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Multiple protein sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and analyzed on DNAMAN 6.0. The signal peptide was predicted using the Signal IP 3.0 server (http://www.cbs.dtu.dk/services/SignalIP/). MEGA 5.10 was used to construct phylogenetic trees. The web-based SMART program (http://smart.embl-heidelberg.de/) was used to detect the protein domains, while the molecular weight and isoelectric point of both SP3 and SP5 were obtained through ExPASy (http://www.expasy.org/tools/protparam.html).

2.4. Co-immunoprecipitation assays

Drosophila S2 cells were used to perform the functional analysis of TRAF6 and SPs. The S2 cells were cultured at 27 °C in Schneider's Drosophila Medium (Serum-Free Medium, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA). Cells were co-transfected with pAc5.1/V5-HisA + TRAF6-HA and pAc5.1/V5-HisA + SP3-His or pAc5.1/V5-HisA + SP5-Flag using the FuGENE®HD Transfection Reagent (Promega, USA). At 36 h post-transfection, immunoprecipitation (IP) assay with anti-HA antibodies (Transgen Biotech., Beijing, China) was carried out using Pierce Co-immunoprecipitation (Co-IP) Kit (Thermo Scientific, USA) according to the manufacturer's instructions, followed by Western blot analysis with anti-HA, anti-Flag or anti-His antibodies (Transgen Biotech., Beijing, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Transgen Biotech., Beijing, China) secondary antibodies were used. Immunoblots were visualized with BeyoECL Plus (Beyotime Biotechnology, Shanghai, China) on AI600 films (GE Healthcare).

2.5. Dual luciferase reporter assays

To determine whether SP3 and SP5 could activate the promoter of ALFs, the pAC5.1-V5-HisA–SP3 (or -SP5) constructed plasmid was co-transfected with the luciferase reporter vectors (pGL3–Basic, pGL3–ALF1, pGL3–ALF2, pGL3– ALF3, pGL3– ALF4, pGL3– ALF5 and pGL3–ALF6, respectively). The pRL-TK Renilla luciferase vector was used as an internal control. *Drosophila* S2 cells were seeded onto 96-well culture plates in 100 μL medium at 2×10^5 cells mL^{-1} 24 h prior to transfection. At 36 h post-transfection cells were harvested and lysed for dual luciferase activity determination using the dual luciferase reporter assay system (Promega, USA).

2.6. Quantitative RT- PCR analysis

Quantitative RT- PCR (qRT-PCR) was carried out using the SYBR® Premix Ex Taq™ II Kit (Takara, Dalian, China) on a LightCycler® 480 system (Roche, USA). The total reaction volume was 20 μL containing 10 μL of SYBR® Premix Ex Taq™ II, 2 μL of 4-fold diluted cDNA, 0.8 μL (10 μM) each of forward and reverse primers (Table 1), and 6.4 μL of ultra-pure water. The amplification program included a denaturation step of 95 °C for 30 s, and 40 cycles of 95 °C for 5 s, 60 °C for 20 s, followed by a melting curve analysis from 65 °C to 95 °C. Each sample was

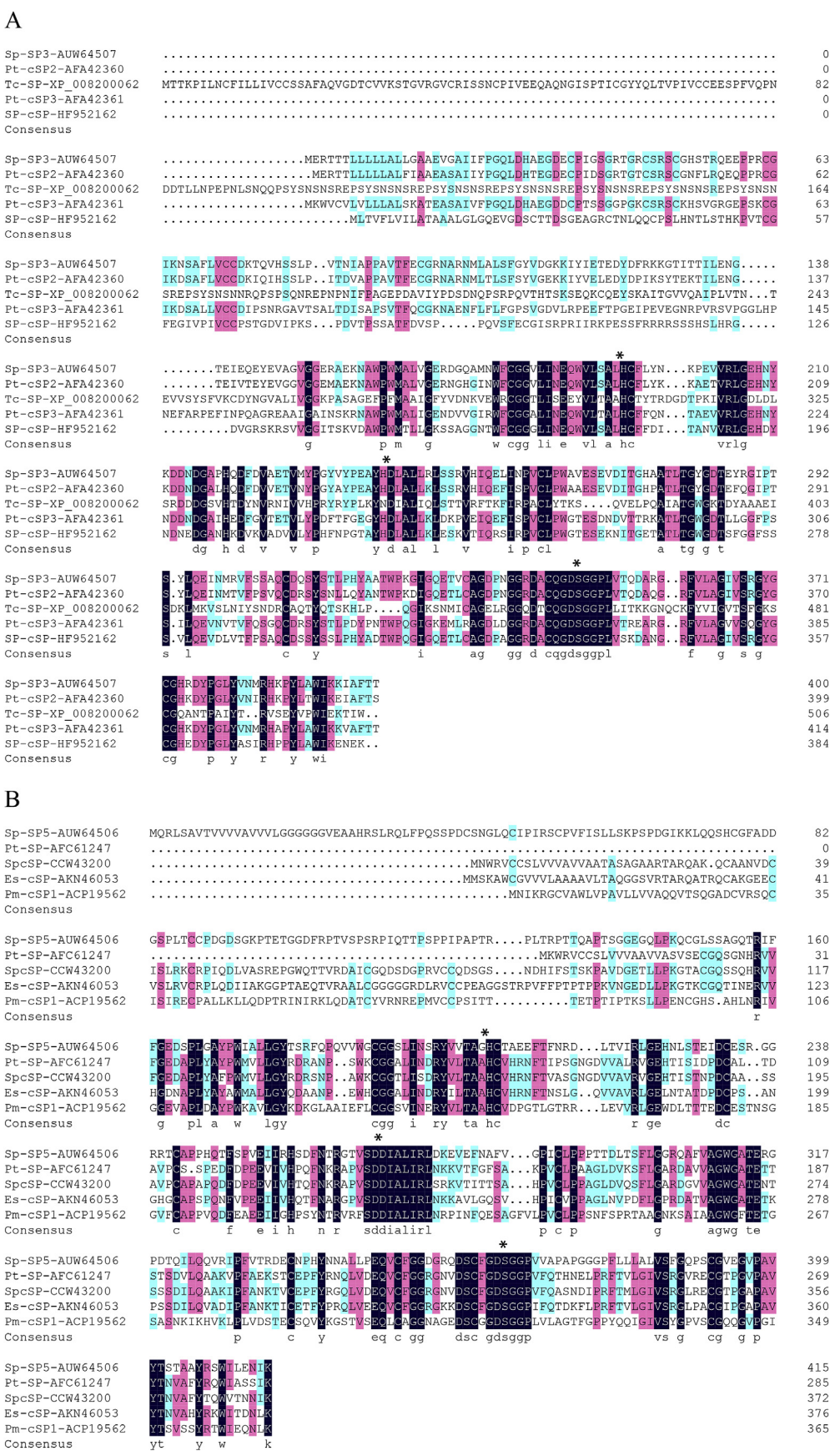

Fig. 1. Multiple sequence alignments of SP3 and SP5 protein with other known SPs. High, medium and low conserved amino acid residues are enclosed in black, violet and light blue, respectively. The catalytic residues at the active site are with an asterisk, and dots (.) indicate gaps. *Sp*-SP3, *S. paramamosain* SP3 (MF973063); *Sp*-SP5 *S. paramamosain* SP5 (AUW64506); *Pt*-cSP2, *Portunus trituberculatus* cSP2 (AFA42360); *Pt*-cSP3, *P. trituberculatus* cSP3 (AFA42361); *SP*-cSP *S. paramamosain* cSP (HF952162); *Tc*-SP *T. castaneum* SP (XP_008200062); *Pt*-SP *P. trituberculatus* SP (AFC61247); *Spc*SP *S. paramamosain* cSP (CCW43200); *Es*-cSP *E. sinensis* cSP (AKN46053); *Pm*-cSP1 *P monodon* cSP1(ACP19562). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

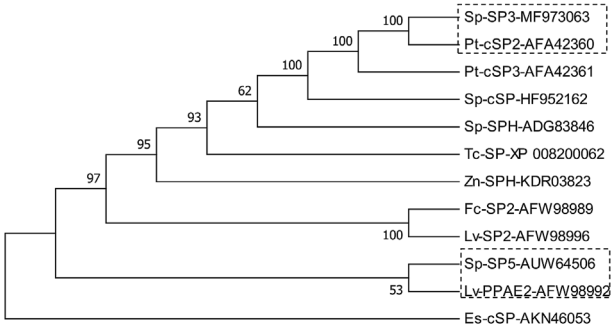

Fig. 2. Phylogenetic tree analysis of SP3 and SP5. 1000 bootstraps were performed on the Neighbor-joining trees to check repeatability of the results using the MEGA 5 software. *Sp*-SP3, *S. paramamosain* SP3 (GenBank accession number: MF973063); *Sp*-SP5, *S. paramamosain* SP5 (AUW64506); *Pt*-cSP2, *Portunus trituberculatus* cSP2 (AFA42360); *Pt*-cSP3, *P. trituberculatus* cSP3 (AFA42361); *Sp*-cSP *S. paramamosain* cSP (HF952162); *Sp*-SPH, *S. paramamosain* SPH (ADG83846); *Tc*-SP, *Tribolium castaneum* SP (XP_008200062); *Zn*-SPH, *Zootermopsis nevadensis* SPH (KDR03823); *Fc*-SP2, *Fenneropenaeus chinensis* SP2 (AFW98989); *Lv*-SP2, *L. vannamei* SP2 (AFW98996); *Lv*-PPAE2 *L. vannamei* PPAE2 (AFW98992); *Es*-cSP, *Eriocheir sinensis* cSP (AKN46043).

analyzed in triplicate. After the PCR program, data were analyzed using the LightCycler 480 software (Roche, USA). The relative transcript levels of SP3 and SP5 were determined using the $2^{-\Delta\Delta Ct}$ algorithm with β-actin as the internal control [34]. All data were expressed as means ± SE. The efficiency of the amplification of the target transcript or the β-actin was also detected. Data were subjected to one-way ANOVA analysis using Origin Pro 8.0 followed by *t*-test, and *P*-values less than 0.05 were considered statistically significant and extremely significant at $P < 0.01$.

2.7. Prokaryotic expression and protein purification

The SP3 and SP5 genes were amplified using the primers E-SP3-F/E-SP3-R and E-SP5-F/E-SP5-R, respectively, followed by purification and double digestion of the PCR products with the restriction endonucleases *Eco*R I and *Xho* I (NEB, USA) at 37 °C overnight. Next, the digested PCR products were cloned into a pGEX-6P-1 plasmid and transformed into Rosetta-gami™2 (DE3) plysS competent cells (Novagen, Germany). Positive clones were identified by PCR with primers pGEX5′ and pGEX3'. Positive transformants and negative controls were both grown in 300 mL LA medium (100 μg mL^{-1} ampicillin) at 37 °C at 200 rpm to an OD_{600} of 0.5–0.7. Isopropyl-β-D-thio-galactoside (IPTG) was then added to the medium to a final concentration of 0.15 mM (positive transformant) or 0.1 mM (negative control) to induce protein expression, and the culture incubated at 16 °C at 140 rpm for 16 h.

Cells were harvested by centrifugation at 6000 × *g* for 5 min at 4 °C, re-suspended in 1 × PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4) including 1% Triton- X-100, and sonicated at 4 °C for 20 min with a sonicator (BILON-250Y) set at 3 s sonication and 4 s interval under 60% power. Cell lysates were centrifuged at 10 000 × *g* for 15 min at 4 °C to collect the supernatant (containing glutathione *S*-transferase), and then ProteinIso® GST Resin (TransGen Biotech, Beijing, China) was added to purify the proteins. The purified proteins were then examined using 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and visualized with Coomassie brilliant blue R250. The concentrations of recombinant SP3 (rSP3), recombinant SP5 (rSP5) protein and the glutathione *S*-transferase (GST) were quantified with bicinchoninic acid (BCA) Protein Assay Kit (Qiagen, Germany). A monoclonal anti-GST antibody (Transgen Biotech, Beijing, China) was used to confirm the proteins and their molecular weights.

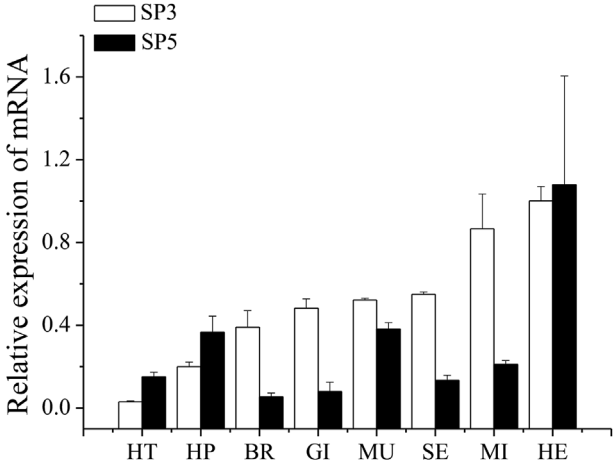

Fig. 3. Tissue distribution of SP3 and SP5 transcripts. Vertical bars represent mean ± S.E (n = 3) for each tissue. Each bar represents the mean value from three determinations with standard error. SP3 and SP5 transcript levels in various tissues were normalized to that in hemocytes (β-actin as the reference gene for internal controls). HE: hemocytes; MI: mid-intestine; HP: hepatopancreas; MU: muscle; SE: subcuticular epidermis; GI: gill; BR: brain; HT: heart.

2.8. Antibody preparation

The purified recombinant SPs were used for antiserum preparation. First, five Balb/c mice were immunized four times in the first week with 100 μg of rSP3 (or rSP5) protein that was thoroughly mixed with Freund's Complete Adjuvant (MP Biomedicals, USA). Following this, 50 μg highly purified rSP3 (or rSP5) thoroughly mixed with Freund's Incomplete Adjuvant (MP Biomedicals, USA) were injected two times once per week. A booster injection was administered a week later. Finally, mice sera were collected seven days after the final immunization and stored at −80 °C.

2.9. RNA interference

The small interfering RNAs (siRNA), SP3-siRNA1-4 (siSP3), SP5-siRNA1-4 (siSP5), TRAF6-siRNA1-4 (siTRAF6) and siGFP1-4 (Table 1), were synthesized using *in vitro* Transcription T7 Kit (Takara, Dalian, China). Concentrations of the dsRNAs were determined by a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA), and the integrity of the products was determined by electrophoresis on a 1.2% agarose gel. Next, laboratory-acclimated mud crabs were injected with 50 μg siSP3, 50 μg siSP5 or 25 μg siTRAF6 into the fourth leg of each crab, while the negative control group crabs, were each injected with an equivalent amount of siGFP. To ascertain the efficiency of the knockdown, hemocytes were collected from three mud crabs per group at 24 h post siSP3 and siTRAF6 injection or at 48 h post siSP5 injection, and used to determine SP3, SP5 and TRAF6 expression by qRT-PCR using the primer pairs Q-siSP3F and Q-siSP3R, Q-siTRAF6F and Q-siTRAF6F, Q-siSP5F and Q-siSP5F. The relative mRNA expressions of SP3, SP5, and TRAF6 were determined using the $2^{-\Delta\Delta Ct}$ algorithm with β-actin as the internal control. The data were subjected to one-way ANOVA analysis using OriginPro 8.0 followed by *t*-test, and *P*-values less than 0.05 were considered statistically significant and extremely significant at $P < 0.01$.

2.10. Western blot analysis

After the RNAi, the SP3 and SP5 protein levels were determined by Western blot. Hemocytes were collected and then treated with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China). Proteins were separated on 10% SDS-PAGE and transferred onto polyvinylidene

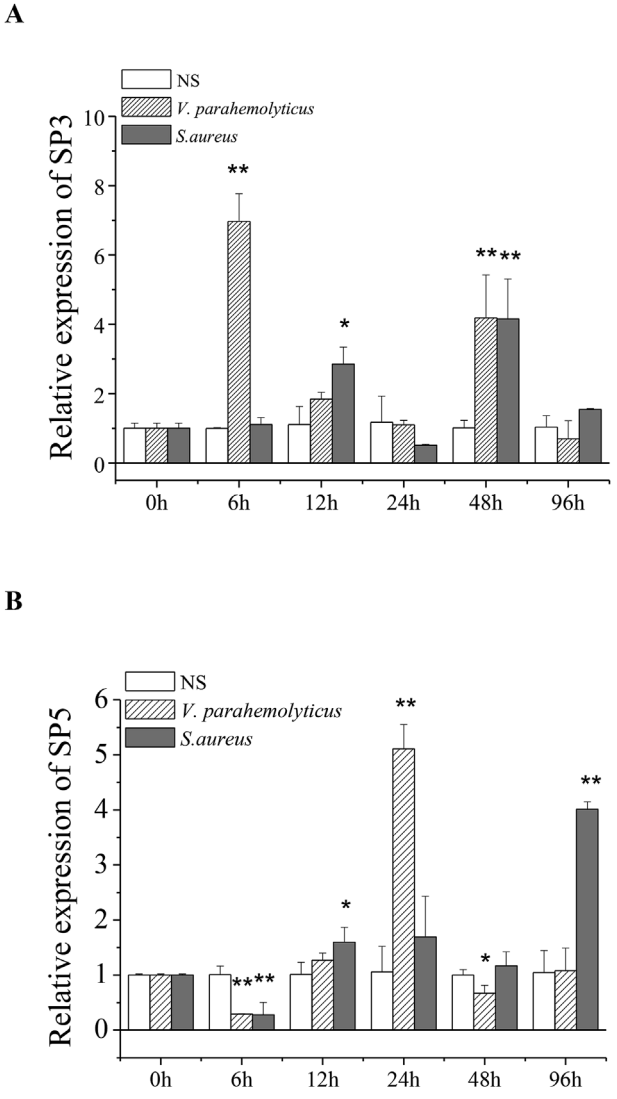

Fig. 4. The expression profiles of SP3 and SP5 after immune challenges. Total RNA was extracted from mud crab hemocytes at different time points after *Vibrio parahemolyticus* and *Staphylococcus aureus* challenges. Samples challenged with NS (0.8% NaCl) were used as control and β-actin as the internal controls. Data is shown as mean ± sd. Significance was compared between the treatment and the control groups at the same time point. Asterisks indicate significant differences (*$P < 0.05$ and **$P < 0.01$).

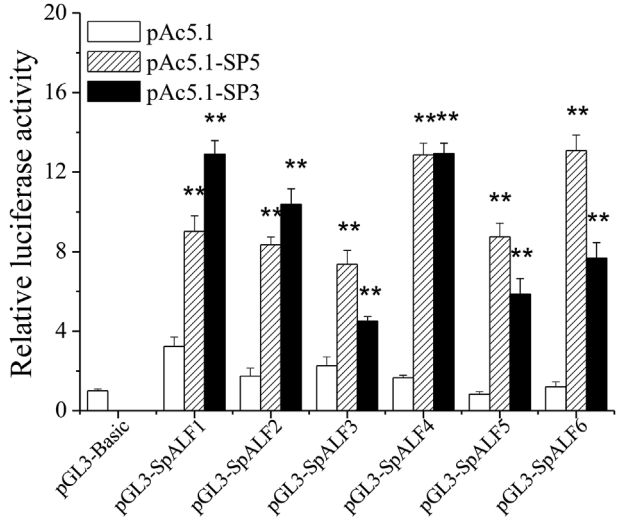

Fig. 6. Dual-luciferase reporter assay in S2 cells. pGL3-Basic: blank control. pAc5.1: native control, pAc5.1/V5-HisA. pGL3-SpALFx: where x = 1, 2, 3, 4, 5, and 6 represented the pGL3-SpALFs recombinant luciferase reporter vectors. pAc5.1-SP3 or pAc5.1-SP5: experimental group. All data were normalized to pGL3-Basic and renilla luciferase activities as an internal reference. Data was shown as mean ± S.E of the luciferase activity (n = 3). Statistical significance is indicated by asterisks (*$P < 0.05$ and **$P < 0.01$).

fluoride (PVDF) membranes. Membranes were incubated with mouse anti-SP3 or anti-SP5 polyclonal antibody, and horseradish peroxidase (HRP)-conjugated goat anti-mouse (Transgen Biotech., Beijing, China) as a secondary antibody. Tubulin was used as an internal reference protein. Antibody binding complexes were visualized with BeyoECL Plus (Beyotime Biotechnology, Shanghai, China) on AI600 films (GE Healthcare).

2.11. Analysis of the expression patterns of immune related genes in mub crab

A total of 16 mud crabs (approximately 35 g each) were divided into four groups, with two crabs from each group injected with siSP3 or siSP5 (50 μg crab^{-1}) while the remaining were injected with siGFP. At 24 and 48 h post injection, hemocytes from at least three mud crabs per group were sampled. Total RNA was extracted, reverse transcribed into first-strand cDNA, diluted 3-fold and used as templates for qRT-PCR. The expression patterns of six ALFs (*Sp*ALF1 to *Sp*ALF6) of mud crab in each group were analyzed using primer pairs *Sp*ALF1F and *Sp*ALF1R, *Sp*ALF2F and *Sp*ALF2R, *Sp*ALF3F and *Sp*ALF3R, *Sp*ALF4F and *Sp*ALF4R, *Sp*ALF5F and *Sp*ALF5R, *Sp*ALF6F and *Sp*ALF6R, *Sp*TRAF6-F and *Sp*TRAF-R, *Sp*proPOF and *Sp*proPOR, Q-siTRAF6F and Q-siTRAF6R, Q-siSP3F and Q-siSP3R, Q-siSP5F and Q-siSP5R, Sp-cSPF and Sp-cSPR,

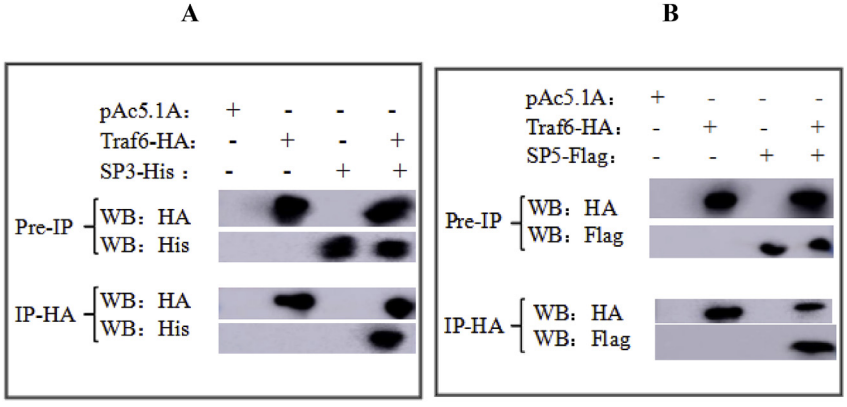

Fig. 5. Co-immunoprecipitation of SP3 and SP5 with TRAF6 in S2 cells. Cells were co-transfected with plasmids expressing TRAF6-HA, SP3-His, and SP5-Flag. A quarter of the cell extract was subjected to the pre-IP assay to assess TRAF6-fusion, SP3-fusion, and SP5-fusion protein levels. The rest of the extract was subjected to IP assay. The IP was analyzed by immunoblot assay probing with anti-HA, anti-His, and anti-Flag antibody.

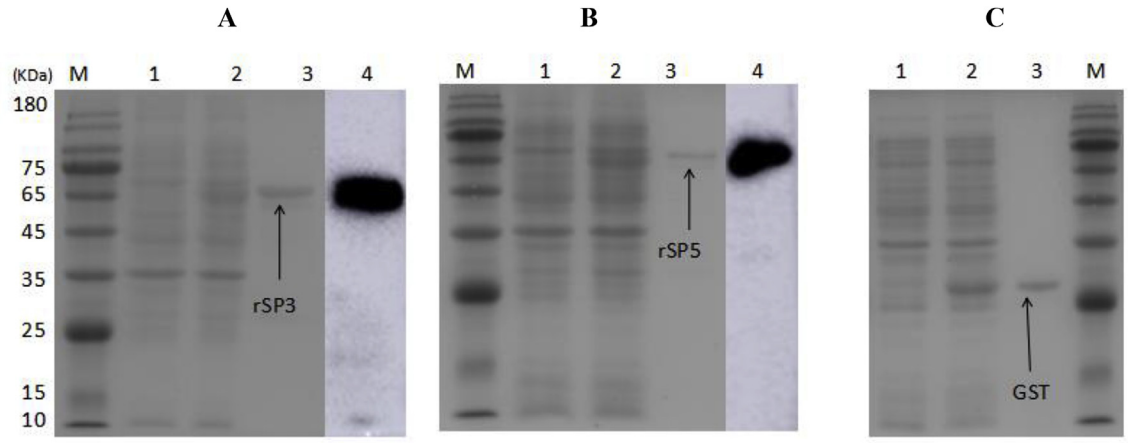

Fig. 7. SDS-PAGE analysis of rSP3 (A), rSP5 (B) and GST (C). The arrows show rSP3, rSP5, and GST. Lane M: standard protein marker; lane A1: total protein obtained from *Escherichia coli* expressing pGEX-6p-1-SP3 without induction; lane A2: total protein of *E. coli* with pGEX-6p-1-SP3 induced with 0.15 mM IPTG; lane A3: purified recombinant SP3; lane A4: identified rSP3 by western blot; lane B1: total protein obtained from *E. coli* expressing pGEX-6p-1-SP5 without induction; lane B2: total protein of *E. coli* with pGEX-6p-1-SP5 induced with 0.15 mM IPTG; lane B3: purified recombinant SP5; B4: identified rSP5 by western blot lane C1: negative control for GST (without induction); lane C2: 0.1 mM IPTG induced GST; lane C3: purified GST.

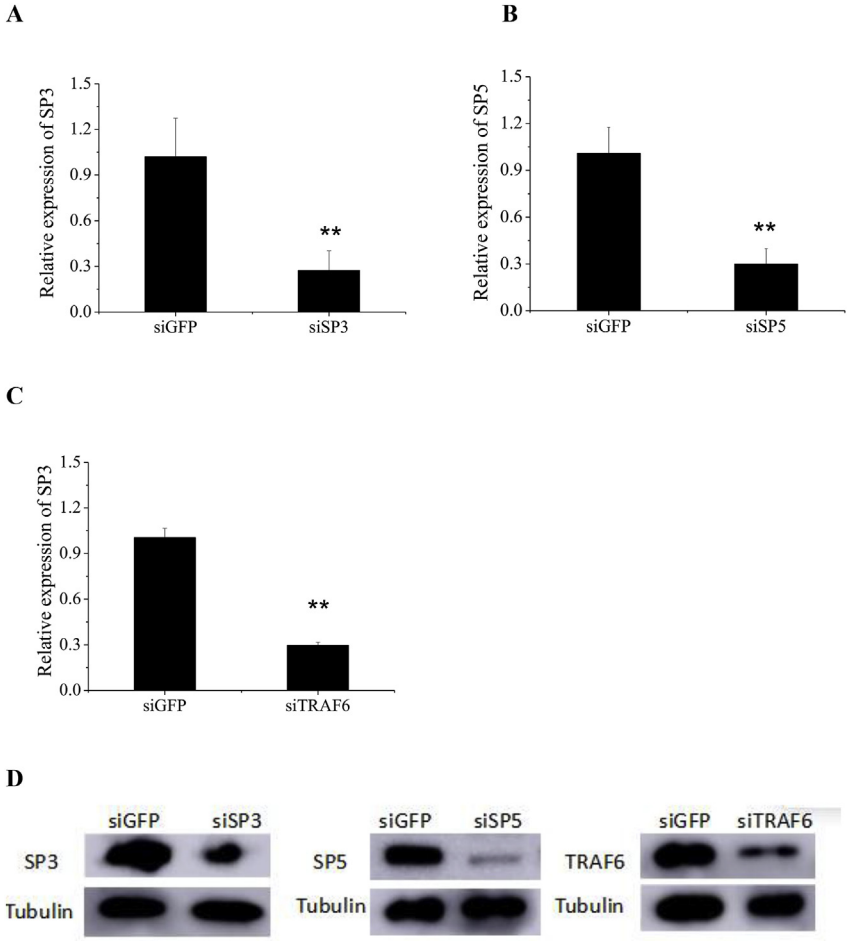

Fig. 8. The interference efficiency of SP3, SP5, and TRAF6 in mud crab hemocytes. The mRNA expression of SP3 in hemocytes after injection with siSP3-48 h, SP5 in hemocytes after injection with siSP5-24 h and TRAF6 in hemocytes after injection with siTRAF6-24 h, determined by qRT-PCR (A, B and C). All data were normalized to siGFP treated samples and β-actin as an internal reference. Data was shown as mean ± S.E. Significance was compared between the treatment and the control groups at the same time point. Asterisks indicated significant differences (*$P < 0.05$ and **$P < 0.01$). (D) Protein expression of SP3 after SP3 RNAi, SP5 after SP5 RNAi and TRAF6 after TRAF6 RNAi. Hemocytes were collected after injected siSP3-48 h, siSP5-24 h and siTRAF6-24 h, siGFP groups were used as the controls.

SpcSPF and SpcSPR, SP4F and SP4R respectively (Table 1). Analyses were repeated at least three times with β-actin used as the internal control. All data were expressed as means ± S.E and analyzed as described above.

2.12. Hemolymph PO activity of mud crab

Mud crab hemocytes collected at 24 h after siSP3 and siTRAF6 or at 48 h after siSP5 injection were used for phenoloxidase (PO) assay. Total protein concentration was measured using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Briefly, hemocytes collected from 1 mL of hemolymph was diluted with 435 μL 10 mM Tris-HCL (pH 8.0) and then mixed with 65 μL dopamine (3 mg mL^{-1} water) (Solarbio, Beijing, China). The reaction mixture was incubated at room temperature for 30 min and stopped by adding 500 μL 10% acetic acid. The PO activity was monitored by

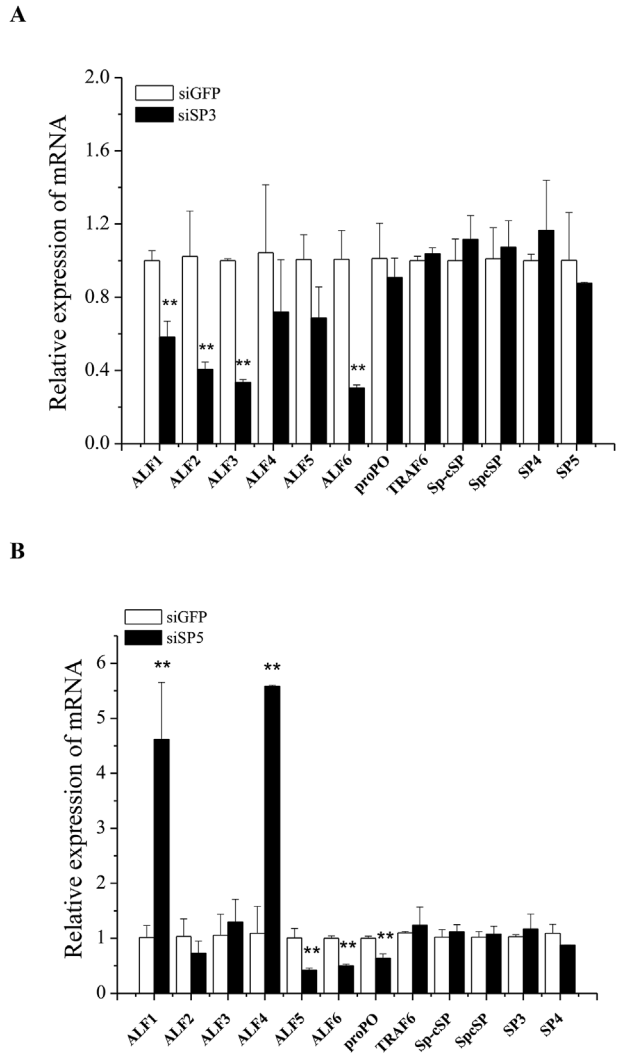

Fig. 9. The expression patterns of immune related genes in mud crab hemocytes. Following treatment with siRNA (A and B) mRNA expression level of immune related genes were detected by qRT-PCR at 48 h after SP3 RNAi or 24 h after SP5 RNAi. Data was shown as mean ± S.E and β-actin as the reference gene for internal controls. Significance was compared between the treatment groups and the control groups at the same time point. Asterisks indicated significant differences (*$P < 0.05$ and **$P < 0.01$).

spectrophotometry at 470 nm to follow the enzyme reaction, from dopamine quinone to melanochrome [35]. The PO activity was recorded as A470/mg total protein/min against control. The experiment was repeated at least three times.

3. Results

3.1. Characterization of SP3 and SP5

The complete cDNA sequence of mud crab SP3 has a length of 1366 bp, containing a 5′-untranslated region (5′-UTR) of 32 bp, an open reading frame (ORF) of 1206 bp, encoding 401 deduced amino acids, and a 128 bp 3′-UTR with a poly(A) tail (Fig.S1 A). The sequence has been deposited at NCBI GenBank under the accession number MF973063. The sequence of SP3 has a typical *N*-terminal signal peptide (residues 1–21) with the putative protein sequence containing a serine proteinase like C-terminal domain (residues 148–393) with a typical catalytic triad formed by three amino acids (H_{193}, D_{241}, and S_{347}). The protein was estimated to have a molecular weight of 44155.81 Da with a theoretical isoelectric point of 5.34. Similarly, the complete cDNA sequence of mud crab SP5 has a length of 1655 bp, containing a 5′-UTR of 36 bp, ORF of 1251 bp, encoding 416 deduced amino acids, and a 368 bp 3′-UTR with a poly(A) tail (Fig.S2 B). The sequence has also been deposited at GenBank under the accession number AUW64506. The sequence of SP5 consists of a typical *N*-terminal signal peptide (residues 1–26) and a clip domain (residues 40–90). The putative protein sequence contains a serine proteinase like C-terminal domain (residues 158–410) with a typical catalytic triad formed by three amino acids (H_{205}, D_{269}, and S_{364}). The molecular weight of the putative protein is 44104.83 Da and has a theoretical isoelectric point of 5.84.

3.2. Sequence and phylogenetic tree analysis

The amino acid sequences of mud crab SP3 and SP5 were aligned with those of other crustaceans (Fig. 1A). SP3 displayed the highest identity of 82% with *Pt*-cSP2 (AFA42360) from *Portunus trituberculatus*, and a relatively moderate identity with the other SPs, such as 59% with *Pt*-cSP3 (AFA42361) from *P. trituberculatus*, 54% with *Sp*-cSP (HF952162) from *S. paramamosain,* and 37% with *Tc*−SP (XP_008200062) from *T. castaneum.* On the other hand, SP5 shares 50% identity with *Pt*-SP (AFC61247) from *P. trituberculatus*, 41% with *Sp*cSP (CCW43200) from *S. paramamosain* and *Es*-cSP (AKN46053) from *E. sinensis*, 40% identity with *Pm*-cSP1 (ACP19562) from *P. monodon*. The results also revealed that a trypsin-chymotrypsin (Tryp_SPc) domain (in all SPs) and three catalytic triad residues (SP3 and SP5) was conserved across species (Fig. 1A and B). A phylogenetic tree for SP3 and SP5 was constructed using the Neighbor-joining method with SP homologs of crustaceans (Fig. 2). The results revealed that mud crab SP3 is closer to *P. trituberculatus Pt*-cSP2 (AFA42360), while SP5 is closer to *L. vannamei Lv*-PPAE2 (AFW98992) phylogenetically.

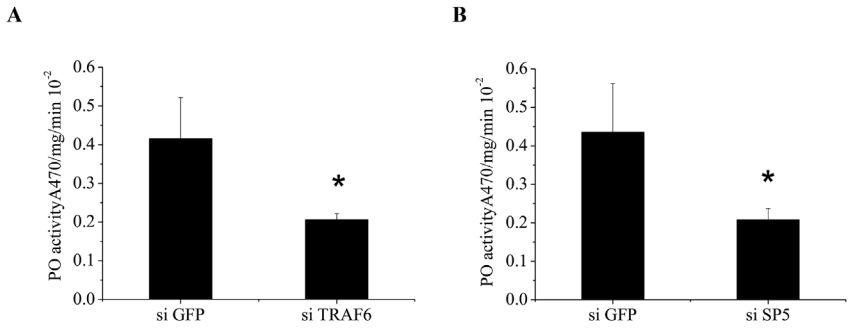

Fig. 10. Knockdown of SP5 and TRAF6 in mud crab hemocytes and total hemolymph PO activity. The PO activity was measured by spectrophotometry at 470 nm and recorded as A470/mg total protein/min. The data from three independently replicated experiments were shown as the means ± S.E. Asterisks indicated significant differences (*$P < 0.05$ and **$P < 0.01$).

3.3. Tissue distribution of SP3 and SP5 in healthy mud crab

The tissue distribution of SP3 and SP5 in eight tissues (hemocytes, gill, mid-intestine, hepatopancreas, muscle, subcuticular epidermis, brain, and heart) from healthy mud crab was analyzed (Fig. 3). The results showed that SP3 and SP5 were constitutively expressed in all examined tissues, with both SPs mainly expressed in hemocytes, while SP3 was lowly expressed in the heart and SP5 in the brain.

3.4. Expression profiles of SP3 and SP5 after V. parahemolyticus and S. aureus challenges

The responses of SP3 and SP5 to *V. parahemolyticus* and *S. aureus* challenges were investigated in mud crab hemocytes at different time points (0, 6, 12, 24, 48 and 96 h) post challenge using qRT-PCR (Fig. 4). The results revealed that SP3 was upregulated at 6 and 48 h following *V. parahemolyticus* challenge, but at 12 and 48 h with *S. aureus* challenge (Fig. 4A). On the other hand, the expression of SP5 was downregulated at 6 and 48 h following *V. parahemolyticus* challenge, followed by a sharp upregulation at 24 h, and returning to baseline at 96 h. For *S. aureus* challenge, the expression of SP5 was downregulated at 6 h and upregulated at 12 and 96 h (Fig. 4B). These results indicated that both SP3 and SP5 might be involved in the immune response of mud crab to bacterial infection.

3.5. SP3 and SP5 interact with TRAF6

Co-IP assays were performed using *Drosophila* S2 cells co-expressing HA-tagged TRAF6, His-tagged SP3 and Flag-tagged SP5 (Fig. 5). There was co-expression of TRAF6-HA with either SP3-His or SP5-Flag in S2 cells, indicating that both SP3-His and SP5-Flag were co-immunoprecipitated by TRAF6-HA. This suggests a specific interaction between TRAF6 and SP3 or SP5.

3.6. Overexpression of SP3 or SP5 activated the promoters of ALFs in S2 cells

The interplay between TRAF6 and SP3 or SP5 in regulating the expression of ALFs *via* the TLR/NF-κB pathway was examined by dual luciferase reporter assays. The results showed that SP3 induced a 3.99-fold, 5.97-fold, 2-fold, 7.87-fold, 7.09-fold, and 6.37-fold overexpression of mud crab ALFs (*Sp*ALF1, *Sp*ALF2, *Sp*ALF3, *Sp*ALF4, *Sp*ALF5, and *Sp*ALF6, respectively) (Fig. 6). Similarly, SP5 enhanced the overexpression of *Sp*ALF1, *Sp*ALF2, *Sp*ALF3, *Sp*ALF4, *Sp*ALF5, and *Sp*ALF6 by 2.97-fold, 4.81-fold, 3.27-fold, 7.76-fold, 10.58-fold, and 10.87-fold, respectively (Fig. 6). These results demonstrated that SP3 and SP5 regulated the activation of ALFs through the TLR/NF-κB pathway.

3.7. Expression and purification of recombinant rSP3 and rSP5

The recombinant plasmid pGEX-6P-1-SP3 and pGEX-6P-1-SP5 were transformed and expressed in Rosetta-gami^{TM}2 (DE3) plysS cells. After IPTG induction, the recombinant proteins (rSP3 and rSP5) expressed in the supernatant were analyzed by SDS-PAGE, which showed, two sharp bands (ca. 69 kDa and ca. 70 kDa) corresponding to the putative molecular mass of the fusion proteins (Fig. 7A and B). On the other hand, the pGEX-6P-1 vector had a distinct 26 kDa band, representing GST (Fig. 7C). The purified recombinant proteins were confirmed by SDS-PAGE and Western blot.

3.8. Knockdown of SP3, SP5 and TRAF6 expression

RNAi was employed to investigate the functions of SP3, SP5, and TRAF6 in mud crab. First, the knockdown efficiency of SP3, SP5 and TRAF6 were determined using qRT-PCR and Western blot. The qRT-PCR results showed that the mRNA expression of SP3, SP5, and TRAF6 were decreased by 70%, 75%, and 71%, respectively (Fig. 8 A, B, and C). To ascertain the knockdown efficiency at the protein level, Western blot analysis was also carried out after siRNA injection. The results showed that compared with the control siRNA, injection of siSP3, siSP5 and siTRAF6 attenuated SP3, SP5 and TRAF6 protein expression in hemocytes of mud crabs (Fig. 8D).

3.9. SP3 and SP5 modulate the expression of immune related genes in mud crab

RNAi was carried out to determine whether SP3 and SP5 participated in regulating ALFs (*Sp*ALF1-6) and proPO gene expression (Fig. 9). The transcript levels of different ALFs (*Sp*ALF1-6) and other immune related genes were determined in mud crab hemocytes after SP3 and SP5 knockdown. Following SP3 knockdown, there was significant down-regulation of *Sp*ALF1, *Sp*ALF2, *Sp*ALF3 and *Sp*ALF6 in mud crab hemocytes, with no significant down-regulation in the expression of *Sp*ALF4, *Sp*ALF5, SP4, SP5, Sp-cSP, SpcSP, TRAF6 and proPO. Similarly, after SP5 knockdown, the transcript levels of *Sp*ALF1 and *Sp*ALF4 were up-regulated, while the mRNA levels of *Sp*ALF5, *Sp*ALF6, and proPO were down-regulated, with no significant change in the expression of *Sp*ALF2 SP3, SP4, Sp-cSP, SpcSP, TRAF6 and *Sp*ALF3.

3.10. SP5 and TRAF6 modulates the PO activity in hemolymph

To verify whether SP3, SP5, and TRAF6 were involved in the proPO system, the PO activity was measured by recording the A470 mg^{-1} total protein min^{-1} (Fig. 10). The results showed that the PO activity decreased by 52.5% and 52.8% after siSP5 and siTRAF6 injection, respectively, compared to the controls. However, there was no significant decrease in the PO activity after SP3 knockdown (data not shown). These results indicate that the SP5 and TRAF6 are involved in the regulation of the proPO system in mud crab.

4. Discussion

The Toll-MyD88-Tube/Pelle-TRAF6 type of signaling pathway is important in the immune system of organisms, as it participates in the regulation of bacterial infections by positively modulating downstream innate immune-related genes [33,36–39]. TRAF6 is an important adaptor protein involved in the nuclear factor-kappa B (NF-κB) signaling pathway. Previous studies have shown that TRAF6 is a molecular bridge that links the upstream TLRs, MyD88, and IRAKs with the downstream NF-κB and mitogen activated kinase (MAPK)-signaling pathways [40]. In this study, we focused on the interaction between TRAF6 and two serine proteases (SP3 and SP5) in the hemocytes of mud crab. Our results showed that both SP3 and SP5 specifically interacted with the TRAF6 protein, indicating that they might play important roles in the immune system of mud crab against pathogen invasions.

Previous studies of SPs in invertebrates have shown that they are important in various innate immune responses, including apoptosis, AMPs synthesis, antimicrobial and antiviral activities, blood clotting, and melanization [14,18,41–44]. Many SPs have been identified in invertebrates, including five SPs and four SPHs in *P. trituberculatus* [45–48], and two SPs and one SPH in *S. paramamosain* [14,16,44]. In addition, SPs and their homologs have also been found in a number of insects, including *B. mori* (15 SPs), *Manduca sexta* (42 SPs), *A. mellifera* (44 SPs and 13 SPHs), *D. melanogaster* (45 SPs), and *A. aegypti* (63 SPs) [18,49–53]. Our current results have revealed the existence of two SPs in mud crab, which were involved in the biological process of the host.

In this study, two novel SPs (SP3 and SP5) were identified and characterized in mud crab. Our results show that both SP3 and SP5 are very similar to homologs from other crustaceans, with a Tryp_SPc domain at the C-terminal and have the three conserved catalytic triad residues. SP5 shares the highest homology with *L. vannamei Lv*-PPAE2,

suggesting that they probably have the same functions in the activation of the prophenoloxidase system [54]. Both SP3 and SP5 transcripts were detected in all examined tissues, including heart, muscle, hepatopancreas, brain, skin, gill, mid-intestine, and hemocytes, which is synonymous with previous observations in *S. paramamosain* [44], *Scylla serrata* [55]. The high expression of SP3 and SP5 observed in mud crab hemocytes suggests that they might be involved in defense against pathogenic invasions [44,56].

The expression in response to challenge by *V. parahaemolyticus* and *S. aureus* were investigated. An upregulated expression of SP3 was observed at 6 and 48 h and at 12 and 48 h after challenges with *V. parahemolyticus* and *S. aureus*, respectively. For SP5, a significantly increase in expression at 24 h, but down-regulation in expression at 6 and 48 h was observed with *V. parahaemolyticus* challenge, while an upregulated expression at 12 and 96 h with *S. aureus* but downregulated at 6 h. Similar results have been reported in *P. trituberculatus* [45,48]. These results indicate that both SP3 and SP5 play a crucial role in regulating the immunity of the mud crab against microbial infections.

ALFs are efficient immune effectors that play important roles in the innate immunity of invertebrates, and were first isolated from hemocytes of horseshoe crab [57]. The results revealed that both SP3 and SP5 could interact with TRAF6 and stimulated the expression of ALFs simultaneously in mud crab. Our results were similar to a previous study where the silencing of TRAF6 in mud crab hemocytes inhibited the expressions of ALF1, ALF2, ALF5 and ALF6 [33]. Interestingly, depletion of SP3 or SP5 suppressed the expression of ALFs (*Sp*ALF1, *Sp*ALF2, *Sp*ALF3, and *Sp*ALF6 or *Sp*ALF5 and *Sp*ALF6), while overexpression of SP3 or SP5 enhanced the activation of these ALFs (*Sp*AFL1-6). In order to make sure the specificity of the siRNA (SP3 and SP5) the expression analysis of other SP isoforms (Sp-cSP, SpcSP and SP4) and TRAF6 be performed and they have no obvious changes. This observation suggests that both SP3 and SP5 are involved in the regulation of ALFs through immune signaling pathway. Moreover, SP3 was found to be more effective than SP5 in the regulation of the ALFs synthesis. However, further studies are required to confirm the roles of ALFs in the TLR-TRAF6-NF-κB signaling pathway. The observations here are similar to previous findings in other invertebrates. For instance, AMPs genes in *P trituberculatus Pt*ALFs and *Pt*Crustin were expressed when *Pt*cSP2 was suppressed [43]. Similarly, the production of AMPs was regulated by clip-SPs through the activation of the Toll pathway in *D. melanogaster* and *M. sexta* [18,25].

An important characteristic of the innate immune system of arthropods is the activation of the SP cascade pathways in hemolymph, which induces activation of proPO in a manner similar to the vertebrate complement system, as it leads to the formation of toxic quinones and melanin [58]. In crustaceans, several studies have revealed vital role of melanization in defense against microbial infections [59,60]. The mRNA level of proPO was significantly decreased following SP5 knockdown (but not by SP3). This observation is similar to previous studies where the expression of proPO was markedly decreased with *Sp*cSP silencing at 6 h [44]. When the functional interactions between SP3 or SP5 and TRAF6 was further examined, it was found that the hemolymph PO activity decreased by 52.5% and 52.8%, respectively, following silencing of SP5 and TRAF6, while SP3 depletion did not significantly reduce the PO activity. These results indicated that both SP5 and TRAF6 might be associated with regulation of proPO activation. Similar results had previously been reported for the SPs in *P. monodon* [35,61] and *Sp*-SPH of *S. paramamosain* to be involved in the activation of the proPO system [16]. Besides, the relationship between the Toll-like pathway and melanin formation (in proPO pathway) has been reported in silkworm (*B. mori*) [62]. These two pathways are activated by hemolymph protease 6 in *M. sexta* after challenge with either bacteria or fungi [49,63]. These results suggest that both SP5 and TRAF6 be able to modulate the PO activity in the hemolymph of mud crab.

In conclusion, two novel SPs (SP3 and SP5) from the mud crab were identified and characterized. Both SP3 and SP5 are involved in the immune response of mud crab to bacterial infection (i.e. *V. parahemolyticus* and *S. aureus*). Our findings revealed that both SP3 and SP5 interacted with TRAF6 and were associated with the activation of ALFs through the TLR/NF-κB pathway. SP5 was less effective than SP3 in the regulation of the ALFs synthesis, but important in proPO activation. Furthermore, both SP5 and TRAF6 regulate PO activity in the hemolymph of mud crab. While these are interesting findings, the factors influencing the activation of the proPO system in mud crabs require further investigations.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.10.014.

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