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Functional characterization of two clip-domain serine proteases in the swimming crab *Portunus trituberculatus*

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ABSTRACT

Clip domain serine proteases (cSPs), a family of multifunctional proteins, play a crucial role in innate immune system. Here, we report the functional characterization of two clip domain serine proteases (PtcSP1 and PtcSP3) from the swimming crab *Portunus trituberculatus*. The recombinant N-terminal clip domains and the C-terminal SP-like domains of PtcSP1 and PtcSP3 were expressed in *Escherichia coli* system, and assayed for various biological functions: protease activity, antimicrobial activity, bacterial clearance and microbial-binding activity. The recombinant SP-like domains of PtcSP1 and PtcSP3 exhibited trypsin-like protease activity, while their recombinant clip domains showed strong antibacterial activity and could bind to bacteria and yeast, suggesting the potential roles of PtcSP1 and PtcSP3 in immune defense and pattern recognition. Unlike PtcSP3, PtcSP1 revealed the opsonic activity as shown by a higher bacterial clearance rate of *Vibrio alginolyticus* coated with the combination of the recombinant clip domain and SP-like domain of PtcSP1 as compared with *V. alginolyticus* only. Knockdown of PtcSP1 or PtcSP3 by RNA interference resulted in a significant decrease of total phenoloxidase (PO) activity in crab, suggesting that PtcSP1 and PtcSP3 are involved in the proPO system. In addition, suppression of PtcSP1 or PtcSP3 changed the expression of PtALFs and complement-like components. All these findings suggest that PtcSP1 and PtcSP3 are multifunctional immune molecules and perform different protective functions in crab defense.

1. Introduction

The innate immunity, generally divided into cellular and humoral responses, provides the first line of defense against microbial infections [1–3]. In invertebrate, the cellular responses consist of phagocytosis, encapsulation and nodule formation, whereas the humoral responses include clotting system, synthesis of antimicrobial proteins, and activation of prophenoloxidase (proPO) system [4–8]. The proPO system is important to arthropods, and is activated by limited proteolysis of serine proteases (SPs) to produce the melanin and toxic reactive intermediates against invading pathogens [3,9].

SPs are one of the largest enzymes families with wide species distribution [10], and participate in regulating diverse physiologic processes, such as food digestion [11], embryonic development [12], hemolymph coagulation [13] and defense responses [14]. The clip-domain SPs (cSPs) belong to members of SPs and consist of the clip domain at the N-terminus and the SP-like domain at the C-terminus [15]. The clip domain is a 37–55 amino acid sequence knitted by three-disulfide bonds [9], while the proteolytic SP-like domain is characterized by a conserved domain (Tryp_SPc domain) with the His–Asp–Ser catalytic triad [10]. Most cSPs in crustaceans are reported to function in the proPO activating system, such as PmClipSP2 and PmPPAE2 in the black tiger shrimp *Penaeus monodon* [16,17], LvPPAE1 in the white shrimp *Litopenaeus vannamei* [18], and EscSP and EsCDSP in the Chinese mitten crab *Eriocheir sinensis* [14,19].

In addition to their role in proPO system, many cSPs and cSP homologs (cSPHs) in insects have been shown to be involved in production of antimicrobial peptides (AMPs) and regulation of the complement-like components [15,20,21]. For example, two cSPs, Grass in *Drosophila* and SAE in *Tenebrio molitor*, could regulate the synthesis of AMPs by the activation of the Toll pathway [22,23]. A common serine protease called Spatzle processing enzyme (SPE) was proposed to

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mediate the molecular cross-talk between Toll signaling pathway and proPO system in *Drosophila* [24]. Moreover, in *Anopheles gambiae*, two cSPHs SPCLIP1 and CLIPA2 could act as key regulators of the complement-like pathway by regulating the accumulation or consumption of complement C3-like protein TEP1 [20,21]. However, the function of cSPs in regulating crab immune signaling remain poorly understood.

In our previous study, two clip domain serine proteases, designated as PtcSP1 and PtcSP3, were identified from the swimming crab *Portunus trituberculatus* [25]. To further investigate the biological function of PtcSP1 and PtcSP3, the recombinant N-terminal clip domains (rPtcSP1-N and rPtcSP3-N) and the C-terminal SP-like domains (rPtcSP1-C and rPtcSP3-C) proteins were expressed and purified in this study. The purified recombinant proteins were carried out (1) to test serine protease activity, (2) to detect their potential effects on bacterial and fungal growth, (3) to examine microbial-binding activity, and (4) to measure the bacterial clearance activity. Furthermore, the involvement of PtcSP1 and PtcSP3 in the proPO system, the regulation of the complement-like components and the synthesis of AMPs was clarified by siRNA or dsRNA mediated RNA interference (RNAi).

2. Materials and methods

2.1. Cloning of N-terminal clip domain and the C-terminal SP-like domain of PtcSP1 and PtcSP3

The N-terminal clip domain (PtcSP1-N, PtcSP3-N) and C-terminal SP-like domain (PtcSP1-C and PtcSP3-C) of PtcSP1 and PtcSP3 were separately amplified based on the four pairs of gene-specific primers ClipcSP1F and ClipcSP1R, SPcSP1F and SPcSP1R, ClipcSP3F and ClipcSP3R, SPcSP3F and SPcSP3R (*Bam* HI and *Xho* I sites were underlined, Table 1). The PCR was performed in a 25 μL reaction volume consisting of 19.3 μL sterile distilled H_2O, 2.5 μL of 10 × PCR buffer, 0.5 μL of dNTP (10 mM), 0.75 μL of each primer (5 mM), 0.2 μL (1 U) of Taq polymerase (TaKaRa), and 1 μL of DNA template (approximately 30 ng). The PCR temperature profile was 94 °C for 3 min, followed by 34 cycles of 94 °C for 30 s, 50 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis, purified and cloned into pMD19-T simple vector (TaKaRa).

2.2. The construction of recombinant plasmids

After being transformed into the competent cells of *Escherichia coli* Trans1-5α (TransGen), the positive recombinants were identified through anti-Amp selection and PCR screening with M13 primers and the specific primers (Table 1). The plasmids containing PCR products or pET-32a (+) vector were digested with the restriction enzymes *Bam* HI and *Xho* I (NEB), then the PCR products were inserted into the *Bam* HI and *Xho* I sites of pET-32a (+) plasmid to generate pET-32a-PtcSP1-N, pET-32a-PtcSP1-C, pET-32a-PtcSP3-N and pET-32a-PtcSP3-C recombinant plasmids. The recombinant plasmids were firstly transformed into the competent cells of *E. coli* Trans1-5α (TransGen). Positive clones were screened by PCR reaction with specific primers and T7 primers and confirmed by nucleotide sequencing. Then the plasmids containing PCR products were transformed into the competent cells of *E. coli* BL21 (DE3)-pLysS (TransGen) and the positive clones were screened as above. The pET-32a (+) vector without insert fragment was selected as a negative control, which could express a thioredoxin (Trx) with 6 × His-tag in the prokaryotic expression system.

2.3. Expression and purification of recombinant PtcSP1-N, PtcSP1-C, PtcSP3-N and PtcSP3-C proteins

Positive transformants of recombinant proteins and negative control were incubated in 200 mL LB medium (containing 100 μg/mL ampicillin) at 37 °C with shaking at 220 rpm. When the culture reached OD_{600} of 0.5–0.7, isopropyl-b-D-thiogalactosidase (IPTG) was added to the final concentration of 1 mM, and incubated for another 4 h under the same conditions. After centrifugation, the cells were resuspended in buffer I (50 mM sodium phosphate, 300 mM NaCl, pH 7.0), sonicated at 4 °C for 30 min. The cell lysate and inclusion bodies were separated by the centrifugation. The inclusion bodies were washed once with buffer I, twice with buffer II (50 mM sodium phosphate, 300 mM NaCl, 2 M urea, pH 7.0) and dissolved in buffer III (50 mM sodium phosphate, 300 mM NaCl, 8 M urea, pH 7.0). The recombinant proteins and negative control sample (rTrx) were purified by cobalt affinity chromatography (Clontech) as described by manufacturer under the denatured condition.

The purified proteins were refolded in gradient urea-TBS glycerol buffer (50 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 1% glycine, 1 mM EDTA, 0.2 mM oxidized glutathione, 2 mM reduced glutathione, 6, 4, 3,

Table 1
Primers used in this study.

Name	Sequence (5′-3′)	PCR objective
ClipcSP1F	GGATCCCAAATTATCTTCCCCAATG	Recombinant expression
ClipcSP1R	GAGCTCCTATTGGTCGCCGCACGA	Recombinant expression
SPcSP1F	GGATCCAGAGTCGTTGGAGGACAGGCAA	Recombinant expression
SPcSP1R	GAGCTCATGTAAAGAGAGCTAGGTCAGG	Recombinant expression
ClipcSP3F	GGATCCAGTGCCATTGTATTTCCCGG	Recombinant expression
ClipcSP3R	CTCGAGTTCTCTTCCTGCTTGGGGGTT	Recombinant expression
SPcSP3F	GGATCCGCAGCAATAGGAGCAATTAAC	Recombinant expression
SPcSP3R	CTCGAGAGATGTGGTGAACGCGACCTT	Recombinant expression
PtcSP3_dF	**TAATACGACTCACTATAGGG**GAGGAATTTACACCTGGCGA	dsRNA synthesis
PtcSP3_dR	**TAATACGACTCACTATAGGG**GCCCGATTATCCAAACACCT	dsRNA synthesis
dEGFP-F	**TAATACGACTCACTATAGGG**GACGTAAACGGCCACAAGTT	dsRNA synthesis
dEGFP-R	**TAATACGACTCACTATAGGG**CTTGTACAGCTCGTCCATGC	dsRNA synthesis
PtcSP1-RTF	ACTATGTCCAGCCAGCGTGT	Real-time PCR
PtcSP1-RTR	CTATGAGCGCGAGTCCTTCC	Real-time PCR
PtcSP3-RTF	AAGCCAGTCGAAATACAGGAG	Real-time PCR
PtcSP3-RTR	CAGCATCTCCTTCCCAATTCC	Real-time PCR
PtALF1-3_RTF	ACGACGAGGAGGAGAAAGAGG	Real-time PCR
PtALF1-3_RTR	GGCACTGATGGTGGAAACTGA	Real-time PCR
PtALF4_RTF	GACGCTCTGAAGGACTTTATG	Real-time PCR
PtALF4_RTR	CGCCGAAACGCTTAGAAATAC	Real-time PCR
PtALF5_RTF	TAGTCGTGGTGAGAGGGCAA	Real-time PCR
PtALF5_RTR	CTTTGCTCTCTCATCAGGAC	Real-time PCR
PtALF6_RTF	CATACTCCCGTGAACTCCTA	Real-time PCR
PtALF6_RTR	CAGACCGTGATTTGTGGAG	Real-time PCR
PtALF7_RTF	GCATTTTCTATTTTCCTATCC	Real-time PCR
PtALF7_RTR	GCATGAGTCTTGTATATTTGG	Real-time PCR
Ptα2M1_RTF	TGTGCCTCCTACCGCCTTCC	Real-time PCR
Ptα2M1_RTR	GGTGTCCCTCTCTCTCAACTCATTT	Real-time PCR
Ptα2M2_RTF	TGAGTCTTGCCTGGTCCACTAAT	Real-time PCR
Ptα2M2_RTR	CGGTGAACACAACCTTCTCCTTT	Real-time PCR
PtTEP_RTF	CTCTTCTCGCTGCTTTCCTTCATC	Real-time PCR
PtTEP_RTR	TTTTTGGGACTTTGCCACCACTT	Real-time PCR
PtC1qR_RTF	ACGTGTCTTCAGGGGCGTGT	Real-time PCR
PtC1qR_RTR	GCTCCCGGTCACCTCTCGTG	Real-time PCR
Actin-F	TCACACACTGTCCCCATCTACG	Real-time PCR
Actin-R	ACCACGCTCGGTCAGGATTTTC	Real-time PCR
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing
RV-M	GAGCGGATAACAATTTCACACAGG	Sequencing
T7 promoter	TAATACGACTCACTATAGGG	Sequencing
T7 terminator	GCTAGTTATTGCTCAGCGGT	Sequencing

The *Bam* HI and *Xho* I sites are underlined and T7 RNA polymerase promoter sequence are bolded.

2, 0 mM urea in each gradient, pH 8.0; each gradient at 4 °C for 12 h). The resultant proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and visualized with Coomassie brilliant blue R250. The concentration of recombinant proteins was measured by BCA (bicinchoninic acid) Protein Assay Kit (Beyotime).

2.4. Protease activity assay

The protease activity was measured according to Jitvaropas method [26]. The recombinant proteins were prepared in 50 mM Tris-HCl buffer (pH 8.0) and the serine protease trypsin (bovine pancreas, Sigma), chymotrypsin (type II bovine pancreas, Sigma) were used as positive controls, Tris-HCl buffer and rTrx diluted with Tris-HCl buffer (pH 8.0) were used as blank group and negative control in each assay. The chromogenic *p*-nitroanilide substrates, N-benzoyl-Phe-Val-Arg-*p*-nitroanilide (FVR, 293.6 μM, Sigma) and N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF, 147.3 μM, Sigma) were used as substrates for trypsin and chymotrypsin, respectively. 60 μL of the recombinant proteins (0.404 μM and 4.04 μM), Tris-HCl buffer, rTrx (0.404 μM and 4.04 μM) or positive control enzymes (0.005 μM trypsin and 0.003 μM chymotrypsin, respectively) was preincubated with 10 μL chromogenic *p*-nitroanilide substrates for 15 min at 30 °C in a 96-well plate. Then, the reaction was stopped by the addition of 20 μL of 50% (v/v) acetic acid and the increase in *p*-nitroaniline production was recorded by measuring the absorbance at 405 nm.

2.5. Antimicrobial activity assay

Antimicrobial activity was measured against two Gram-negative bacteria *Vibrio alginolyticus* and *Pseudomonas aeruginosa*, two Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus* and one fungus *Pichia pastoris* using a liquid phase assay modified from that of Rathinakumar et al. [27]. Briefly, bacteria and yeast were grown to mid-logarithmic phase and diluted with Tris-HCl buffer (50 mM, pH 8.0) to 10^3 CFU/mL. In sterile 96-well plate, 50 μL of recombinant proteins in 1/2-fold serial dilution with 50 mM Tris-HCl buffer (pH 8.0) were added into the wells. The wells with 50 μL of Tris-HCl buffer and 50 μL of rTrx diluted with Tris-HCl buffer (pH 8.0) were used as blank group and negative control. And then 50 μL of cell suspension (10^3 CFU/mL) were added into the wells and mixed. The 96-well plates were incubated at 37 °C for 2 h, and 150 μL of medium was added, and then the mixtures were allowed to recover overnight. Absorbance at 600 nm for Gram-positive bacteria or 560 nm for Gram-negative bacteria and fungus of each well was determined using a precision microplate reader. The assay was performed with triplicates in three independent experiments. The data were subjected to analysis of one-way ANOVA using SPSS 19.0 and considered significant when $P < 0.05$. The minimum inhibitory concentration (MIC) value was expressed as the range between the highest concentration of the protein where microorganisms were growing and the lowest concentration that caused 100% growth inhibition [28].

2.6. Assay of binding activity to microorganisms

The specific microbial-binding activity of rPtcSP1-N, rPtcSP1-C, rPtcSP3-N and rPtcSP3-C was tested against the above mentioned bacteria and fungus by the method described by Lee and Söderhäll [29]. Gram-positive bacteria, Gram-negative bacteria and yeast were cultured at LB, TSB or YPD medium to the logarithmic growth phase respectively, and then fixed with 37% formaldehyde by gently shaking at 37 °C for 1 h to destroy the protease activity of microorganisms. 0.5 mL of the cells suspension (3×10^8) with PBS and 0.5 mL of purified protein and negative control sample (rTrx) (final concentration, 1 mg proteins) were mixed and incubated with gentle rocking at 4 °C for 30 min. After centrifugation at 2000 × *g* and 4 °C for 5 min, the supernatant was removed, and the cells were washed twice with PBS. Bound proteins were subsequently eluted with 1 × SDS-PAGE sample loading buffer. Microorganisms incubated with PBS were used as control. The supernatant, washed and eluted fractions were run on 15% (w/v) SDS-PAGE.

2.7. Assay of in vivo bacterial clearance

The bacterial clearance activity of the recombinant proteins could be demonstrated by the bacterial clearance rates [26]. Specimens of healthy *P. trituberculatus* (150 ± 3 g) were purchased from Nanshan market in Qingdao, China, and cultivated in 140 L tanks (7–10 crabs/tank) for one week before treatment. The bacterial clearance assay was tested as described by (Lee and Söderhäll) [29] with modifications. The recombinant protein of Trx diluted with Tris-HCl buffer (pH 8.0) were used as negative control. *V. alginolyticus* (3.2×10^8 cells) were incubated with the combination of the recombinant N-terminal clip domain and C-terminal SP-like domain proteins of the PtcSP1, PtcSP3 and rTrx (1 μM each) at 4 °C for 1 h to obtain *V. alginolyticus* coated with the recombinant proteins. The supernatant was then removed, and the cells were washed twice with PBS. 100 μL of 1×10^5 cells uncoated *V. alginolyticus* (control) or coated *V. alginolyticus* were injected into healthy crab. Treated crabs were kept at 13 °C in aerated water, and 100 μL of hemolymph from five crabs was collected without anticoagulant at different time periods 15, 30, 60, 90, 120, 150, 180 and 240 min), and immediately plated on thiosulfate–citrate–bile–sucrose (TCBS) agar plates incubating at 30 °C for 9 h. The colony forming units (cfu) were counted and the number of viable *V. alginolyticus* in crab hemolymph was calculated at the various sampling times and expressed as cfu mL^{-1} hemolymph. Each experiment was performed in five independent replicates and the effects of time after injection were tested on the following variables using one-way ANOVA followed by Duncan ($P < 0.05$).

2.8. Synthesis of siRNAs and PtcSP1-RNAi assay

Small interfering RNA (siRNA) for RNA interference (RNAi) assays of crab was synthesized *in vitro* using a commercial kit according to the manufacturer's instructions (Takara, Japan). The sequence-specific PtcSP1-siRNA (5′-AAGAAGGTGCTCCTAATCCTG-3′) was synthesized to knock down the expression of PtcSP1 gene, and the sequence of siRNA was scrambled to generate the random-siRNA sequence (5′-GCACUAU CUAGUUGUCGAACA-3′). The formation of double stranded RNAs was monitored by determining the size in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl) and quantified by spectrophotometry. The synthesized siRNA was diluted with 0.1 mol/L PBS to a final concentration of 0.5 μg/μL before injection.

Specimens of healthy *P. trituberculatus* were cultivated as above before treatment. The RNAi assay was conducted in crabs by the injection of siRNA into the third appendage using a syringe, and the amount of injected siRNA was chosen according to our pre-experiment and the RNAi assay in *E. sinensis* [30]. In details, 50 μg of siRNA (PtcSP1-siRNA or siRNA-scrambled) was injected at a volume of 100 μL per crab, and another 50 μg siRNA was injected using the same method at 24 h after the first injection. At the same time, PBS only was injected into crabs as control. For each treatment, the hemocytes from five crabs were collected at 12, 24 and 48 h after the second injection.

2.9. Double strand RNAs (dsRNAs) synthesis and in vivo PtcSP3 knockdown

Forward and reverse primers of PtcSP3 with T7 promoter sequence (Table 1) were designed to amplify the DNA fragment (649 bp) of PtcSP3 based on the cDNA template extracted from hemocytes of *P. trituberculatus*. The products were confirmed by sequencing, used as

templates for synthesis of dsRNA of PtcSP3 with TranscriptAid T7 High Yield Transcription kit (Thermo Fisher Scientific, USA) following the manufacturer's protocols. The dsRNA fragment of the enhanced green fluorescent protein (EGFP, 694 bp) was synthesized based on the template of pEGFP vector with T7 promoter linked primers dEGFP-F and dEGFP-R (Table 1). Synthesized dsRNAs were monitored by determining the size shift on 1.2% agarose gel during electrophoresis, and the concentration of dsRNAs was measured by NanoDrop 1000 spectrophotometer (Labtech, UK) and dissolved in 0.1 M phosphate buffer saline (PBS, pH 7.4) to a final concentration of 1 μg/μL.

Crabs receiving an injection of PtcSP3 dsRNA (1.5 μg/g *P. trituberculatus*) resuspended in 0.1 mol/L PBS at the arthrodial membrane of the last walking leg were used as challenge group, while the individuals received an injection of EGFP dsRNA were used as control group. The injected crabs were returned to the water tanks and five individuals were randomly sampled at the time point of 0, 24, 48 and 72 h post-injection.

2.10. Detection of the efficiency of PtcSP1- and PtcSP3- RNAi assay

The hemolymph were harvested from the last walking leg using a syringe and quickly added to an equal volume of anticoagulant modified Alsever solution (glucose, 2.05 g; citrate, 0.8 g; NaCl, 0.42 g; double distilled water was added to 100 mL). Samples were immediately centrifuged at 2500 × *g*, 4 °C for 10 min to collect the hemocytes. Total RNA from hemocytes was extracted using Trizol reagent according to the manufacture's protocol (Invitrogen) and reverse transcribed to cDNA using M-MLV reverse transcriptase (Promega) and oligo dT.

The efficiency of PtcSP1 and PtcSP3 knockdown was checked using quantitative real-time PCR using two pairs of primers PtcSP1-RTF and PtcSP1-RTR, PtcSP3-RTF and PtcSP3-RTR (Table 1), and the optimum time of gene knockdown was found. The β-actin from *P. trituberculatus*, amplified with primers Actin-F and Actin-R (Table 1), was chosen as reference gene for internal standardization. The amplifications were conducted in triplicates in a total volume of 10 μL containing 5 μL of 2 × SYBR Premix Ex TaqTM (TaKaRa), 0.2 μL 50 × ROX Reference Dye II, 4 μL of the diluted cDNA, 0.2 μL of each primer (10 μM), and 0.4 μL of RNase-free H_2O. The PCR program was 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 31 s. Dissociation curve analysis of the amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with ABI7500 SDS 2.0 software (Applied Biosystems). Fold change for the gene expression relative to controls was determined by the $2^{-\triangle\triangle Ct}$ method [31]. The results were subjected to one-way ANOVA using SPSS 19.0, and the *P* values less than 0.05 and 0.01 were considered statistically significant.

2.11. Assay of hemolymph phenoloxidase activity in PtcSP1 or PtcSP3 knockdown crab

Total PO enzymic activity was assayed at 24 h post siRNA or dsRNA injection. Hemolymph was withdrawn without the use of anticoagulant from the last walking leg of the crab. L-3,4-dihydroxyphenylalanine (L-dopa) dissolved in water was used to detect PO activity in the hemolymph extracts according to Amparyup et al. [32]. Briefly, 2 mg of total hemolymph proteins in 435 μL of Tris-HCl (10 mM, pH 8.0) were mixed with 65 μL of freshly prepared L-dopa (3 mg/mL in water) (Sigma). After incubation at room temperature for 30 min, 500 μL of 10% (v/v) acetic acid was added to each mixture. The PO activity was monitored by measuring the absorbance at 490 nm in precision microplate reader (Emax). The assay was performed in triplicates. All data were subjected to one-way ANOVA using SPSS 19.0.

2.12. The expression patterns of immune-related genes in PtcSP1 or PtcSP3 knockdown crab

In order to know the function of PtcSP1 and PtcSP3 in regulating the expression of antimicrobial peptides (AMPs) and complement-like components, nine immune genes including PtALF1-3 (HM627757, HM627758 and GQ165621), PtALF4 (JF756050), PtALF5 (JF756051), PtALF6 (JF756052), PtALF7 (JF756053), Ptα2M1 (MK076885), Ptα2M2 (MK076887), PtTEP (POR|c98947_g3) and PtC1qR (MK076886) were chosen to detect their expression in PtcSP1 or PtcSP3 knockdown crabs. The sequences of primers used in this section were shown in Table 1. The expression level of the above genes at the determined optimum time (24 h) was measured using real-time PCR as previously described.

3. Results

3.1. Overexpression and purification of the recombinant N-terminal clip domain and the recombinant C-terminal SP-like domain of PtcSP1 and PtcSP3

The recombinant plasmids pET-32a-PtcSP1-N, pET-32a-PtcSP1-C, pET-32a-PtcSP3-N and pET-32a-PtcSP3-C were transformed and expressed in *E. coli* BL21 (DE3)-pLysS. The induced rPtcSP1-N, rPtcSP1-C, rPtcSP3-N and rPtcSP3-C were expressed as insoluble proteins and accumulated in inclusion bodies. They had distinct bands with molecular weight of approximately 34, 47, 35 and 45 kDa, respectively (Fig. 1), which were in accordance with the predicted molecular mass of fusion protein. Meanwhile, the transformant with pET-32a vector was induced and a unique 21 kDa expressed product representing Trx was detected and purified from the IPTG induced whole cell lysate (Fig. S1). The purified and refolded rPtcSP1-N, rPtcSP1-C, rPtcSP3-N and rPtcSP3-C were at the same molecular weight. The concentration of the rPtcSP3-N and rPtcSP3-C protein was 2.02, 1.98, 1.24 and 1.08 mg/mL, respectively.

3.2. Serine protease activity assay

The substrate specificity of SPs is usually determined by the binding pocket residues at the positions 189, 216 and 226 [33]. Sequence and alignment analysis showed three residues (Asp189, Gly216 and Gly226) forming the binding pocket were found in PtcSP1 and PtcSP3 (Fig. S2). The serine protease activity was investigated by various chromogenic substrates for trypsin and chymotrypsin (Fig. 2). The purified rPtcSP1-C and rPtcSP3-C protein had significant protease activities on the hydrolysis of FVR ($P < 0.01$) but not for AAPF. No protease activity was detected in all assays of the recombinant PtcSP1-N and PtcSP3-N proteins.

3.3. Antimicrobial activity assay

Antimicrobial activities and MIC of rPtcSP1-N and rPtcSP3-N were determined and summarized in Table 2. No significant antimicrobial activity of rPtcSP1-C and rPtcSP3-C were observed. The rPtcSP1-N and rPtcSP3-N protein could inhibit all the tested Gram-negative and Gram-positive bacteria. Remarkably, rPtcSP1-N showed the highest activity against Gram-negative bacteria *V. alginolyticus* (MIC value of less than 0.93 μM), while rPtcSP3-N strongly inhibited growth of the Gram-positive bacteria *M. luteus* (MIC value of less than 0.58 μM). No obvious antimicrobial activity was observed against yeast.

3.4. Binding activity to microorganisms

The microbial-binding assay revealed that rPtcSP1-N and rPtcSP3-N were both found in the eluted fractions, whereas no band was detected in the supernatant or the washed fractions (Fig. 3A and C), suggesting

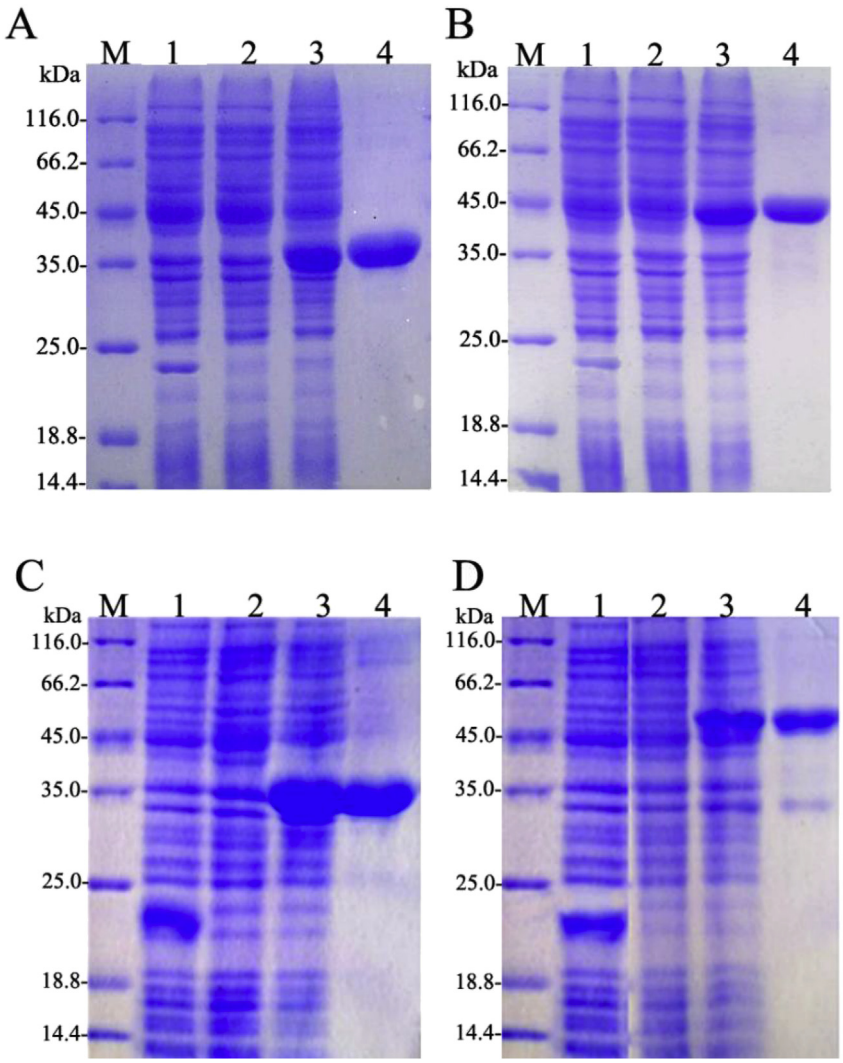

Fig. 1. SDS-PAGE analysis of the recombinant PtcSP1-N (A), PtcSP1-C (B), PtcSP3-N (C) and PtcSP3-C (D) proteins. Lane M: Protein standard; lane 1: IPTG induced rTrx; lane 2: Negative control without IPTG induction; lane 3: IPTG induced recombinant proteins; lane 4: Purified recombinant proteins.

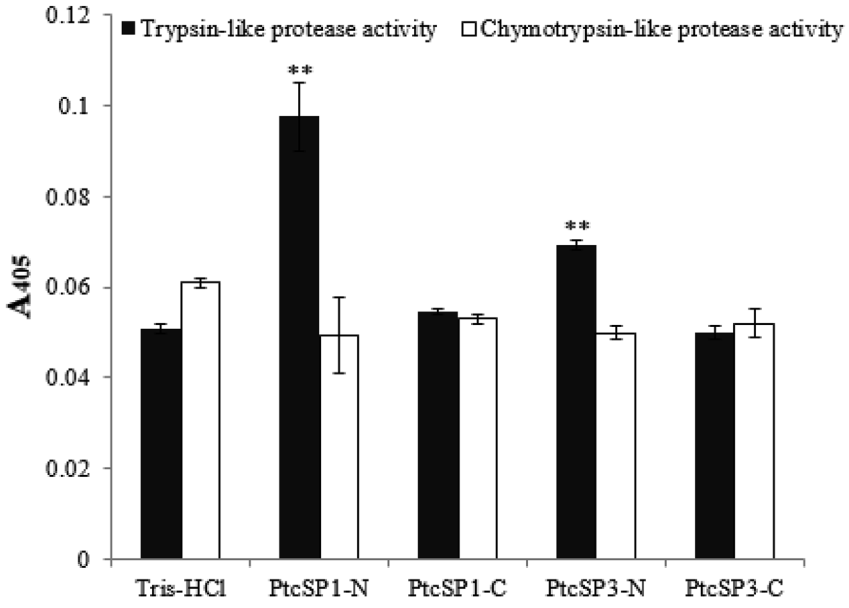

Fig. 2. Serine protease activity of the recombinant PtcSP1-N, PtcSP1-C, PtcSP3-N and PtcSP3-C proteins. Significant differences across Tris-HCl are indicated with two asterisks at $P < 0.01$. PtcSP1-N: the N-terminal clip domain of PtcSP1, PtcSP1-C: the C-terminal SP-like domain of PtcSP1, PtcSP3-N: the N-terminal clip domain of PtcSP3 and PtcSP3-C: the C-terminal SP-like domain of PtcSP3.

Table 2
Antimicrobial activity expressed as minimal inhibition concentrations (MIC) of the recombinant proteins.

Microorganisms	MIC (μM)			
	PtcSP1-N	PtcSP1-C	PtcSP3-N	PtcSP3-C
Gram-negative bacteria				
Vibrio alginolyticus	15.83–31.66	Na	< 0.58	Na
Pseudomonas aeruginosa	7.92–15.83	Na	9.33–18.66	Na
Gram-positive bacteria				
Micrococcus luteus	< 0.99	Na	18.66–37.32	Na
Staphylococcus aureus	1.99–3.98	Na	1.17–2.33	Na
Fungus				
Pichia pastoris	Na	Na	Na	Na

MIC are expressed as the interval a-b, where a is the highest concentration tested at which microorganisms are growing ($P > 0.05$) and b is the lowest concentration that cause 100% growth inhibition ($P < 0.05$). Na: not active up to 63.32 Mm.

that N-terminal clip domain protein could strongly bind to Gram-negative bacteria *V. alginolyticus* and *P. aeruginosa*, Gram-positive bacteria *M. luteus* and *S. aureus* and yeast *P. pastoris*. The recombinant PtcSP1-C and PtcSP3-C protein showed clear bands in the supernatant fractions, and no or weak band in the precipitate fractions (Fig. 3B and D). This results indicated that rPtcSP1-C and rPtcSP3-C might not bind to microorganisms.

3.5. V. alginolyticus clearance from circulation

The opsonic activity of rPtcSP1 and rPtcSP3 were tested under in vivo conditions in crab for their clearance rates compared with that of control bacteria not treated with the recombinant proteins. *V. alginolyticus* (1×10^5 cells) coated with rPtcSP1 (the recombinant N-terminal region and C-terminal SP-like domain proteins) were more rapidly cleared from the crab hemolymph as compared with uncoated *V. alginolyticus* (control) suggested that the PtcSP1 protein functions as opsonic proteins (Fig. 4). No obvious opsonic activity of rPtcSP3 protein was observed (data not shown).

3.6. Gene knockdown

To characterize the potential role of PtcSP1 and PtcSP3 in crab innate immunity, PtcSP1-siRNA and dsPtcSP3 were synthesized successfully and used to challenge the crabs. The relative expression level of PtcSP1 or PtcSP3 in hemocytes after PtcSP1-siRNA or dsPtcSP3 interference was shown in Fig. 5A and B. The most significant effect of PtcSP1 and PtcSP3 were both detected at 24 h post-injection. The silencing efficiency of the PtcSP1-siRNA reached up to 94.8%, while PtcSP3 was interfered by 66.42% with dsPtcSP3. Therefore, 24 h post-dsRNA injection was selected as the optimum time of PtcSP1- or PtcSP3-knockdown and used for further interfering experiments.

3.7. Total PO activity in PtcSP1 or PtcSP3-knockdown crab

To study the possible role of PtcSP1 and PtcSP3 in proPO system, the total PO activity in the hemolymph of PtcSP1 or PtcSP3 knockdown crab was measured. The experiments were repeated three times, and the results showed the significant reduction 33.3% and 31.7% of the total PO activity in the RNAi-mediated PtcSP1 and PtcSP3 knockdown crabs, respectively. (Fig. 6A and B). This results suggested that both PtcSP1 and PtcSP3 might take part in the proPO activation.

3.8. Effects of PtcSP1 or PtcSP3 interference on the expression of complement-like components and AMPs

Under the conditions where the expression of PtcSP1 gene was knocked down, the expression levels of complement-like components genes (Ptα2M2 and PtC1qR) were significantly up-regulated ($P < 0.05$), particularly, Ptα2M2 was dramatically up-regulated to 18.14-fold of that in the control group, while AMP genes (PtALF1-3, PtALF4, PtALF6, PtALF7) was remarkably suppressed ($P < 0.01$). However, the expression of Ptα2M1, PtTEP and PtALF5 was not

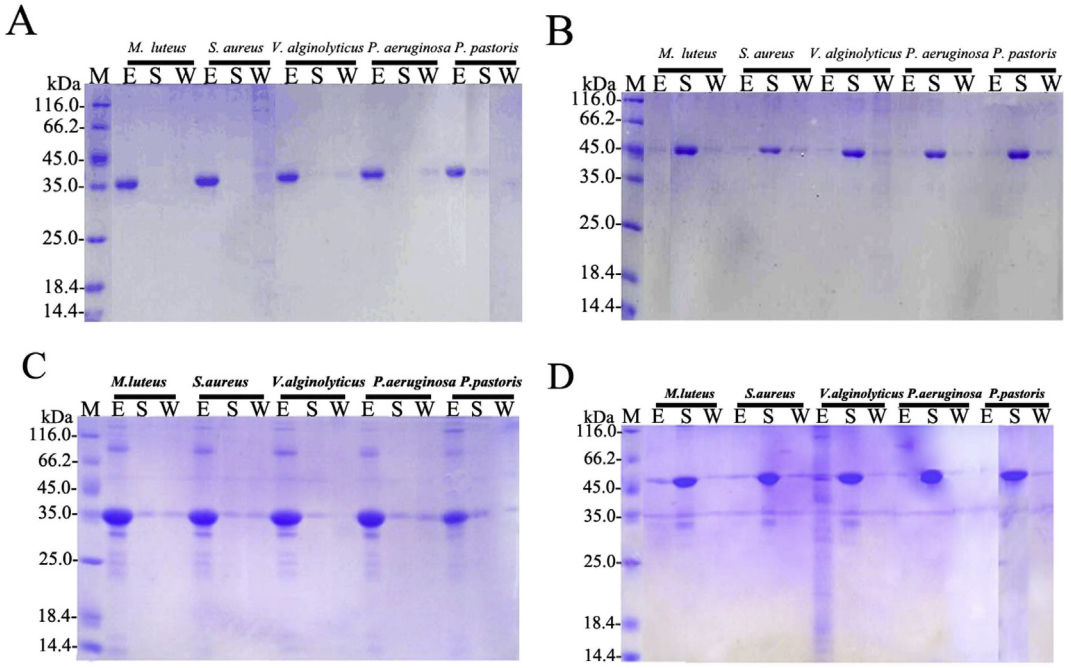

Fig. 3. Binding activity of the recombinant PtcSP1-N (A), PtcSP1-C (B), PtcSP3-N (C) and PtcSP3-C (D) to Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus*, Gram-negative bacteria *Vibrio alginolyticus* and *Pseudomonas aeruginosa*, and yeast *Pichia pastoris*. Initially, the recombinant protein was incubated with formaldehyde-fixed microorganisms. After incubation, the supernatants were separated by centrifugation. The pellets were washed with PBS buffer and the bound proteins were eluted with SDS-PAGE sample loading buffer. The eluted (E), supernatants (S) and washed (W) fractions were examined by SDS-PAGE.

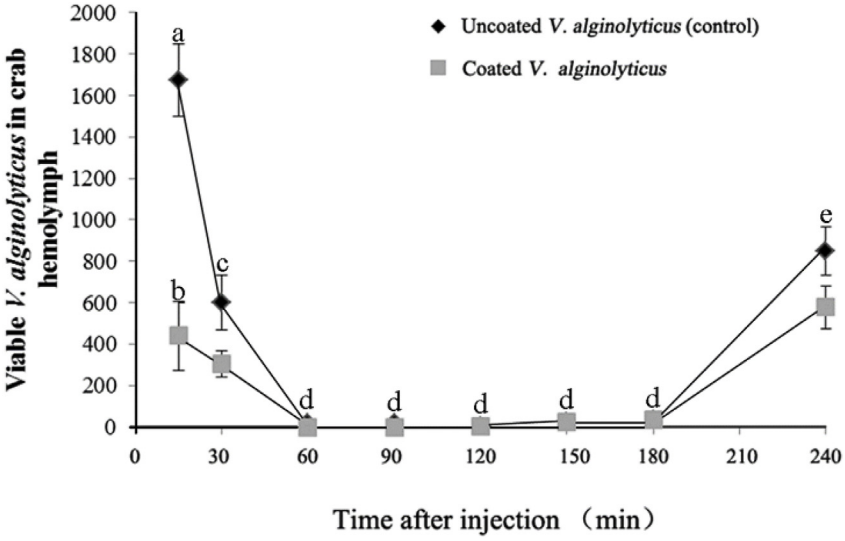

Fig. 4. Clearance rate of *V. alginolyticus* from the hemolymph circulation. Crabs were injected with *V. alginolyticus* coated the recombinant proteins (N-terminal region and C-terminal SP-like domain proteins) or *V. alginolyticus* only (control). The experiment was repeated five times with similar results. Means with the same lower case letters (above each bar) are not significantly different at the $P < 0.05$.

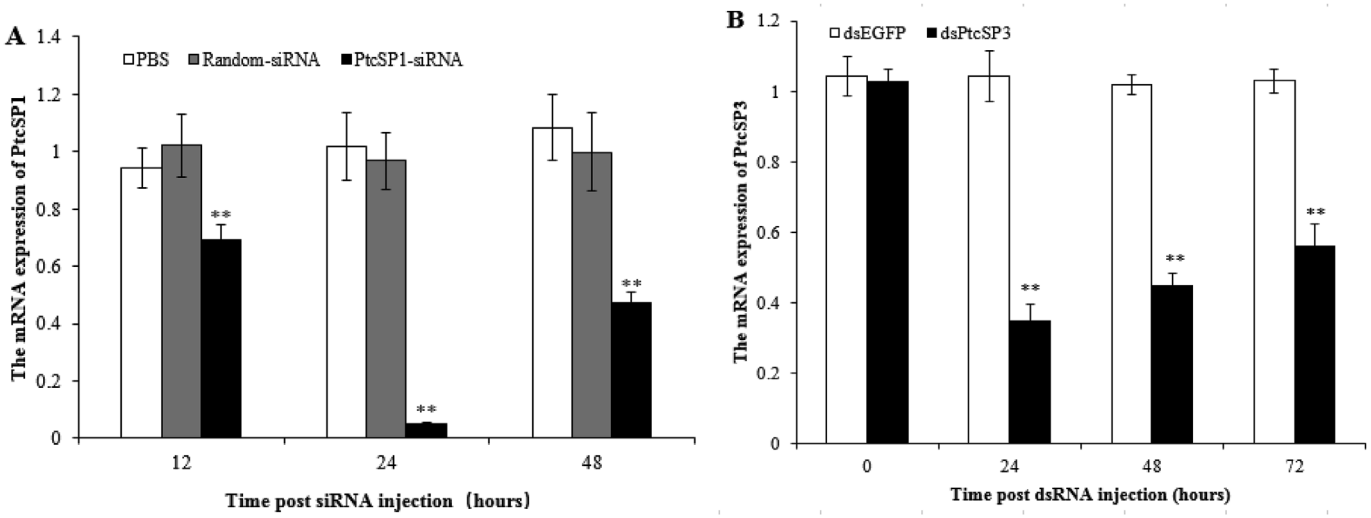

Fig. 5. Expression levels of PtcSP1 (A) and PtcSP3 (B) in crab hemocytes after gene interference. Vertical bars represent the mean ± S.E. (n = 5). Significant differences across control in the same time of sampling are indicated with two asterisks at $P < 0.01$.

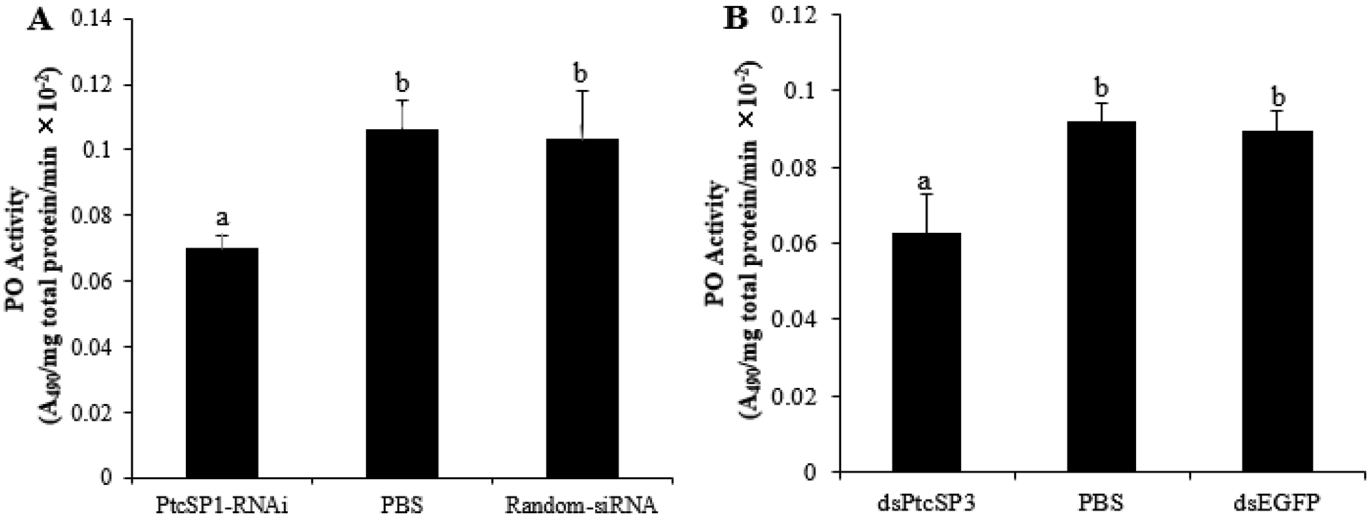

Fig. 6. Total hemolymph phenoloxidase (PO) activity in PtcSP1 (A) or PtcSP3 (B) silenced crab. Experiments were repeated three times and the data is shown as the mean ± standard deviation. Vertical bars represent the mean ± S.E. (n = 5). Means with the same lower case letters (above each bar) are not significantly different at the $P < 0.05$.

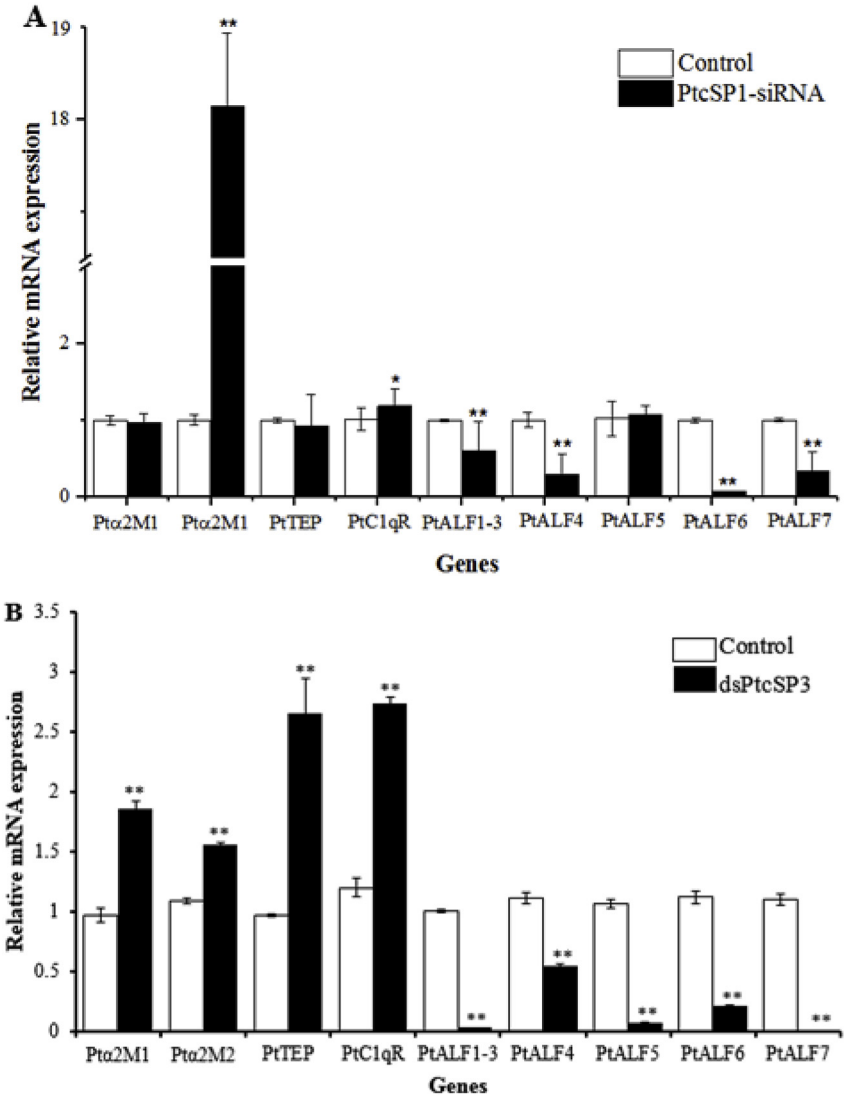

Fig. 7. Expression levels of component-like factors and AMPs after gene silence of PtcSP1 (A) or PtcSP3 (B). Vertical bars represent the mean ± S.E. (n = 5). Significant differences across control in the same time of sampling are indicated with two asterisks at $P < 0.01$.

significantly changed (Fig. 7A). After the injection of dsPtcSP3, the expression of PtALF1-7 was suppressed notably ($P < 0.01$). PtALF7 showed the lowest expression, which was 0.01-fold to that in the control group. On the contrary, complement-like components (Ptα2M1, Ptα2M2, PtTEP and PtC1qR) were apparently up-regulated in the dsPtcSP3 injection group ($P < 0.01$). PtTEP and PtC1qR presented the higher expression with 2.76-fold and 2.28-fold to that in the control group, respectively (Fig. 7B).

4. Discussion

Two cSP genes, namely PtcSP1 and PtcSP3, have been identified and characterized from hemocytes and eyestalk cDNA libraries of the swimming crab *P. trituberculatus* [25]. They are clearly two members of cSP family with a clip domain at the N-terminal and a SP-like domain at the C-terminal. According to their substrate specificity, SPs can be classified into trypsin, chymotrypsin, elastase, collagenase, etc. [11]. PtcSP1 and PtcSP3 have the binding pocket sites Asp189, Gly216 and Gly226 that conserved in trypsin-like cSPs, suggesting they may belong to trypsins. Through prokaryotic expression, the recombinant C-terminal SP-like domain of PtcSP1 and PtcSP3 (rPtcSP1-C and rPtcSP3-C) show high activity on substrate FVR, which further confirm PtcSP1 and PtcSP3 to be trypsin-like cSPs.

Some recombinant clip domains could exhibit antimicrobial activity only against Gram-positive bacteria, such as clip domains of the proPO activating enzyme in *Pacifastacus leniusculus* [34] and PmMasSPH in *P. monodon* [26]. However, in this study, the recombinant N-terminal clip domains of PtcSP1 and PtcSP3 (rPtcSP1-N and rPtcSP3-N) exhibit a broad spectrum inhibition activity toward Gram-negative and Gram-positive bacteria. Similar antibacterial activity is also found in our previous recombinant clip domains of PtcSP and PtcSP2 in *P. trituberculatus* [28,35], demonstrating the direct antimicrobial activity of clip domains of PtcSPs. Moreover, rPtcSP1-N and rPtcSP3-N exist the binding activity to the Gram-negative, Gram-positive bacteria and yeast, suggesting its role in the immune recognition of pathogens. Similar findings have been reported in several cSPs and cSPHs, such as PtcSP2 in *P. trituberculatus* [35], the mas-like protein in crayfish *P. leniusculus* [29], ClipSP2 and c-SPH in shrimp *P. monodon* [16,36]. These results together suggest that PtcSP1 and PtcSP3 may act as potential antibacterial proteins and pattern recognition proteins (PRPs) in crab innate immunity.

The opsonization of invading microorganisms by blood cells contributes to the resistance of various bacteria, and such opsonic effect could be detected by the bacterial clearance [29,37]. In the present study, PtcSP1 exhibits the opsonic activity as demonstrated by a more rapid clearance from the hemolymph of *V. alginolyticus* coated the

combination of the recombinant clip domain and SP-like domain proteins than uncoated control bacteria. Similar clearance of live bacteria has also been reported in mas-like proteins of the shrimp *P. monodon* [26] and the crayfish *P. leniusculus* [29]. However, PtcSP3 shows no obvious bacterial clearance activity, indicating the functional diversity between PtcSP1 and PtcSP3. The viable bacteria could be rapidly cleared from hemolymph by phagocytosis and encapsulation [26,38]. PtcSP1 reveals the opsonic activity as well as cell adhesion and antibacterial activity, which might be greatly accelerate the rate of cellular immune responses.

Si-RNA or double-strand RNA mediated gene interference is used to investigate the potential immune function of PtcSP1 and PtcSP3 on the proPO system or other immune signaling pathways. The current study shows that, the PO activity is significantly decreased in PtcSP1- or PtcSP3-knockdown crabs, suggesting both PtcSP1 and PtcSP3 are proteases that are required for proPO activation. This is consistent with those cSPs reported in the horseshoe crab, the shrimp *P. monodon* and the Asian corn borer *Ostrinia furnacalis* [16,39,40], but in contrast to that observed in PtcSP2-knockdown crabs [35]. Further investigation shows the significant suppression of PtALF1-4 and PtALF6-7 in PtcSP1 knockdown crabs and PtALF1-7 in PtcSP3 silenced crabs. Similar regulation is also found in the serine protease SAE from *T.molitor*, which is required for the induction of AMPs [23]. However, reduction of PtcSP1 and PtcSP2 transcript does not affect the expression of PtALF5 [34]. Taken together, these findings suggest the functional diversity of PtcSPs in the proPO system and the production of AMPs.

Recent studies have demonstrated that some complement-like components, such as mannose-binding lectin (MBL) and alpha-2-macroglobulin (α2M), could involve in activation of the proPO system [41,42]. Our study reveals that the expression of all *P. trituberculatus* complement-like components except Ptα2M1 and PtTEP in PtcSP1 knockdown crabs, are significantly reduced by PtcSP1-siRNA or dsPtcSP3 interference, indicating the negative regulatory roles of PtcSP1 and PtcSP3 in regulating the expressions of complement-like genes. Similar result has been reported in SPH of mosquito *A. gambiae* that CLIPA2 silencing triggered an exacerbated TEP1-mediated response [21]. Moreover, α2M in *P. vannamei* and *L. vannamai* could limit the extent of proPO activity by preventing the trypsin-like SPs from activating the proteinase cascade [42,43], further supporting the negative relationship between SP and α2M. Interestingly, though the expression of Ptα2M1 and PtTEP does not change under the condition of PtcSP1 silenced, Ptα2M2 shows a dramatical up-regulation, suggesting the regulatory specificity of PtcSP1. To our knowledge, this is the first report about the regulation of cSPs on complement-like components in crabs.

In conclusion, our data, including the previous report on PtcSP2 [35], indicate that PtcSP1-3 play important immune roles, probably by exhibiting antibacterial activity, binding to invading pathogens, production of AMP or regulation of complement-like components, in swimming crab defense against invading bacteria. Both PtcSP1 and PtcSP3 are trypsins and participates in the regulation of proPO system, while PtcSP1 participates in the clearance of *V. alginolyticus* but PtcSP3 does not. PtcSP2 is a chymotrypsin and unlikely to be directly involved in the proPO activating system. However, detecting the interacting proteins of PtcSP1-3 and whether PtcSP1-3 interact with each other in crab immune defense requires further investigation.

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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.2018.12.047.

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