

A novel serine protease with clip domain from scallop *Chlamys farreri*

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Received: 17 January 2007 / Accepted: 9 April 2007 / Published online: 6 May 2007
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Abstract The serine proteases with clip domain are involved in various innate immune functions in invertebrate such as antimicrobial activity, cell adhesion, pattern recognition and regulation of the prophenoloxidase system. A serine protease with clip-domain cDNA (Cf SP) was obtained by Expressed sequence taggings (ESTs) method and rapid amplification of cDNA ends (RACE). The Cf SP full-length cDNA was of 1,152 bp, including a 5′-terminal untranslated region (UTR) of 63 bp, a 3′-terminal UTR of 81 bp with a canonical polyadenylation signal sequence AATAAA and a poly(A) tail, and an open reading frame of 1,008 bp encoding a polypeptide of 336 amino acids with a putative signal peptide of 19 amino acids. The deduced amino acid sequence of Cf SP contained an amino-terminal clip domain with three disulfide bonds formed six conserved Cys residues, a carboxyl-terminal trypsin-like domain with the conserved His–Asp–Ser catalytic triad, and a low complexity linker sequence. The Cf SP was strongly expressed in hemocytes and the mRNA expression of Cf

SP was up-regulated and increased 3.2-fold and 2.6-fold at 16 h after injection of *Vibrio anguillarum* and *Micrococcus luteus*. The results suggested that Cf SP gene might be involved in immune response of Gram-negative and Gram-positive microbial infection in scallop

Keywords *Chlamys farreri* · Clip domain · Immune response · mRNA expression · Serine protease · Tissue distribution

Introduction

Chlamys farreri is one of the economic scallop species cultivated widely in the coastal provinces of north China. Since the summer of 1997, large-scale mortality of *C. farreri* has been reported in China and has caused catastrophic economic losses to scallop aquaculture [1]. Although it is still unclear about the causes for the scallop mortality, it is believed that the mortalities are related to the deteriorating water quality, the emerging of diseases and the stock degenerating of inbreeding [2, 3]. Mollusk lack the adaptive immune system that is characteristic of vertebrate organisms, and must rely exclusively on innate immune systems in dealing with a diverse array of pathogens and stress response [4, 5]. Therefore, advances in the characterization of immune regulatory pathways and related immune mediator of scallop will provide a better understanding of the immune defense mechanisms of mollusk and can give new insights into health management and diseases control in scallop aquaculture [6, 7].

Innate immune reactions are initiated when so-called pathogen-associated molecular patterns (PAMPs) of microorganism are detected by pattern recognition receptors (PRRs) of host and recognized as “non-self” [8–11]. Recognition of “non-self” usually activates extracellular

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serine protease cascades that amplify the signal and trigger downstream effector responses, leading to the killing of the invader [11–13]. A novel serine proteases family has already been identified and proved to play critical roles in several defense mechanisms of arthropods and insects [11, 14–17]. For example, in *Drosophila melanogaster*, some serine proteases are key components of the dorsoventral pattern formation system (e.g., Easter and Snake), but others, such as Persephone, are also involved in the activation of the Toll pathway upon fungal infection [17]. In the horseshoe crab *Tachypleus tridentatus* clotting pathway, at least four serine proteases are activated in a cascade manner, leading to the transformation of coagulogen to insoluble coagulum gel and subsequent clotting of the hemolymph [18, 19]. The clot is effective in immobilizing pathogens, which are then eliminated by other host effector mechanisms [19]. In the activation of the prophenoloxidase (proPO) system, serine proteases, known as proPO-activating proteinases (PAPs) or proPO-activating enzymes (PPAEs), have also been isolated from the cuticle of *Bombyx mori* [20, 21], the hemocyte of crayfish [22, 23], the pupae of *D. melanogaster* [24], and the coleopteran insect *Holotrichia diomphalia* [25, 26]. The above serine proteases share common structural features, such as at least one amino-terminal disulphide clip regulatory domain and a trypsinogen-like catalytic domain in the carboxyl-terminus [11, 14, 16].

The clip domain was initially identified in proclotting enzyme from the horseshoe crab *T. tridentatus* and named according to the schematic form of a paper clip [14, 19]. Clip domains are usually composed of 37–55 amino acid residues, which are knitted together by three conserved disulfide bonds and thus are expected to form a quite compact structure [14, 19]. The overall sequence similarity between different clip domains is low, but the six Cys residues are strictly conserved [14]. The role of the clip domain is not yet known, but is believed to involved in protein–protein interactions, regulation of the protease activity, and even antimicrobial activity [14, 27].

Although the biochemical and genetic studies of arthropods and insects immune pathways have demonstrated that serine proteases with clip domain play important roles. However, only a few of serine proteases in mollusk have been completely characterized [28], yet many have not been identified. In order to obtain more information about the genes encoding mollusk serine proteases, the putative serine protease (Cf SP) gene with clip domain was cloned and characterized from the scallop *C. farreri*, and its tissue distribution and temporal expression by microorganism challenge were investigated in the present study.

Materials and methods

Animals, immune challenge and hemolymph collection

Scallops of *C. farreri* (weighting about 25 ± 2 g per scallop), were collected from a commercial farm (Sanggou Bay, Shandong Province, China), and kept into aerated seawater at 15°C for 3 days before processing. Eight hundreds scallops were randomly selected and kept in eight water tanks (100 individuals in each tank) for the challenge experiments. Microbial strains used for challenge were *Vibrio anguillarum*, *Micrococcus luteus* and *Pichia pastoris*, respectively. They were collected by centrifugation (10,000g, 5 min), then washed twice with PBS (pH 6.4, 0.1 mol l^{-1}) and finally re-suspended in PBS to the concentration of $\text{OD}_{600} = 0.4$.

Challenge experiments were performed on the injection of the scallops with 50 μl of *V. anguillarum*, *M. luteus* and *P. pastoris* into the adductor muscles, respectively. Untreated scallops, including infected 50 μl of PBS, were used as the blank group and the control group in the challenge experiments, respectively. The challenged scallops were returned into seawater tanks, and 15 individuals were randomly sampled at 2, 4, 6, 8, 16 and 32 h for subsequent experiments. The hemolymph from the blank, control and the above challenged groups were collected using a syringe from the adductor muscles and centrifuged at 1,000g, 4°C for 10 min to harvest the hemocytes. The pellets of hemocyte were immediately used for RNA extraction.

cDNA library construction and cloning the full-length cDNA of Cf SP gene

A cDNA library was constructed from the whole tissue of a scallop challenged by *V. anguillarum*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene). Random sequencing of the library using T3 primer yielded 6935 successful sequencing reactions. BLAST analysis of all the EST sequences revealed that one EST₃₄₄ was homologous to the mosaic serine protease of epitheliasin (AAF21308) from *Mus musculus* [29] and the clotting factor B (BAA03528) from *T. tridentatus* [19]. Based on the identified EST sequence, two gene-specific primers Cf SP-F and Cf SP-R1 (Table 1) were designed to amplify the full-length cDNA of Cf SP by 5'-RACE and 3'-RACE. The PCR products were gel-purified and sequenced in both directions. The resulting sequences were subjected to cluster analysis.

Table 1 Oligonucleotide primers used in the experiments

Primer name	Sequence	Length (bp)
CFPS2-F	5'-GTAGGAGGCACCACAGCAAC-3'	20
CFPS2-R1	5'-GTTGCTGTGGTGCCTCCTAC-3'	20
CFPS2-R2	5'-GAGTTGCTCCCCATCCAGTTG-3'	21
T3(Vector)	5'-AATTAACCCTCACTAAAGGG-3'	20
T7(Vector)	5'-GTAATACGACTCACTATAGGGC-3'	22
AF	5'-TATGCCCTCCCTCACGCTAT-3'	22
AR	5'-GCCAGACTCGTCGTATTCCT-3'	20

Sequence analysis

The similarity analysis of nucleotide and protein sequence was carried out by using BLAST2.0 at NCBI (<http://www.ncbi.nlm.nih.gov/blast>). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (<http://www.expasy.org/>) and ANTHEPROT 2000 V5.2 software. Multiple alignment of the serine protease gene sequences was performed with the ClustalW Multiple Alignment program (<http://www.ebi.ac.uk/clustalw/>) and Multiple Alignment show program (<http://www.bio-soft.net/sms/index.html>). The clip domain, the proteolytic activation site, catalytic and specificity residues were predicted from comparison with *D. melanogaster* SP sequences [30]. Twenty amino acid sequences of serine protease with clip domain registered in GenBank were used to construct the phylogenetic tree by Neighbor-Joining in Mega 3.1 (Molecular Evolutionary Genetics Analysis Version 3.1) [31] and the accession numbers of the sequences in GenBank are given in the legend to Fig. 2.

Northern blot analysis

Total RNA was extracted from *C. farreri* tissues (adductor muscle, mantle margin, heart, gonad, digestive gland, gill, intestine and hemocyte). RNA samples were denatured and separated by electrophoresis on a formaldehyde/MOPS agarose gel (1.5%) and transferred onto a Positive Charged Nylon Membrane (Roche Applied Sciences). The membrane was UV cross-linked using a GS gene linkerTM UV chamber (Bio-Rad), prehybridized for 3 h in DIG Easy Hyb (Roche Applied Sciences), and incubated with the DIG-labeled Cf SP and beta-actin cDNA probe overnight at 50°C, and then washed twice at 50°C in 0.5× saline sodium citrate (SSC) with 0.1% sodium dodecyl sulfate (SDS). The probe-target hybrids were reacted with anti-DIG, developed with NBT/BCIP (DIG Nucleic Acid Detection Kit, Roche Applied Sciences), and photographed using a digital camera.

Cf SP and beta-actin (used as an internal control) cDNA probes were individually labeled with DIG by PCR

reaction using specific primers (PCR DIG Probe Synthesis Kit, Roche Applied Sciences). The Cf SP cDNA probe (388 bp) was amplified using gene-specific primers Cf SP-F and Cf SP-R2 (Table 1). Beta-actin cDNA probe (751 bp) was produced by PCR with two primers AF and AR (Table 1), which designed based on *C. farreri* beta-actin cDNA sequences (AY335441). To detect the weak transcript of Cf SP gene, the magnitude of RNA and probe for Cf SP was 3-fold than that of beta-actin

Semi-quantitative RT-PCR analysis of Cf SP gene expression

The expression of Cf SP gene after microorganism challenge was measured by the semi-quantitative RT-PCR technique. Total RNA was extracted from the hemocyte pellets using TRIzol reagent (Invitrogen, USA). Single-strand cDNA was synthesized from 1 µg total RNA in a final volume of 20 µl containing 50 pmol random hexamers, 50 mmol l⁻¹ Tris-HCl, pH 8.3, 75 mmol l⁻¹ KCl, 3 mmol l⁻¹ MgCl₂, 50 mmol l⁻¹ DTT, 0.75 U Rnasin, 0.2 mmol l⁻¹ each of dATP, dCTP, dGTP, dTTP and 200 U MMLV reverse transcriptase (Promega). Reactions were incubated at 37°C for 1 h, terminated by heating the mixture at 95°C for 5 min, and subsequently stored at –80°C. Two Cf SP gene-specific primers Cf SP-F and Cf SP-R2 (Table 1) and two beta-actin primers AF and AR were used to amplify 388 bp and 751 bp fragments in the RT-PCR, respectively.

Semi-quantitative RT-PCR was carried out in a PTC-100 Programmable Thermal Controller Cycler (MJ Research) in a 20 µl reaction volume containing 2 µl of 10× PCR buffer, 1.2 µl of MgCl₂ (2.5 mmol l⁻¹), 1.6 µl of dNTP (2.5 mmol l⁻¹), 1 µl of each primer (10 pmol l⁻¹), 13 µl of PCR-grade water, 0.2 µl (1 U) of *Taq* polymerase (Promega) and 1 µl of cDNA reaction mix. The PCR programs was carried out at 94°C for 5 min, followed by 28 cycles (for Cf SP) or 22 cycles (for beta-actin) of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 8 min. The PCR products were separated in 1.5% agarose gel stained with ethidium bromide. To

confirm the specificity of RT-PCR amplification, the RT-PCR products were purified from the gel and submitted for sequencing. Electrophoretic images and the optical densities of amplified bands were analyzed using the software of Scion Image (version B4.02). Statistical analysis was performed with commercially available statistical software (GraphPad Prism v4.0, GraphPad Software). The data were analyzed with a one-way ANOVA followed by the Student-Newman-Keul's test. Differences were considered significant at $P < 0.05$.

Results and discussion

Scallop aquaculture is a big industry and contributes enormously to the economic development of coastal provinces in China. However, the large-scale mortality of *C. farreri* has caused catastrophic economic losses to scallop aquaculture [1]. Understanding the immune defense mechanisms of scallop may be conducive to the development of better disease management strategies in scallop farming [6, 7]. Recent studies have demonstrated in arthropods and insects that serine proteases with clip domain play critical roles in several defense mechanisms, such as the activation of signaling pathways leading to the synthesis of antimicrobial peptides (AMPs) [14, 17], hemolymph agglutination [16, 32] and melanization [11, 15]. However, the molecular characterization and functional studies of serine proteases remains poorly investigated in scallops

In the present study, an 1,152 bp nucleotide sequence representing the complete cDNA sequence of *C. farreri* serine protease (Cf SP) gene was obtained by 5'-RACE and 3'-RACE with specific primers based on the EST₃₄₄ sequence, and the deduced amino acid sequence is shown in Fig. 1. The full-length cDNA of Cf SP contained an open reading frame (ORF) of 1,008 bp encoding a polypeptide of 336 amino acids with a putative signal peptide cleavage site between Ser₁₉ and Gln₂₀. The putative whole protein molecular weight is approximately 35.97 kDa and its putative pI is 6.60, whereas the putative mature protein molecular weight is approximately 33.96 kDa and its putative pI is 6.75.

Structurally, Cf SP has the common features of the clip domain serine protease family: a N-terminal disulfide knotted clip domain (Cys₂₄–Cys₆₀), which stabilized by three disulfide bonds formed between six conserved Cys residues, a C-terminal typical trypsinogen-like catalytic domain (Ile₁₀₅–Ile₃₂₉) with the conserved His–Asp–Ser catalytic triad, and a linking sequence (Tyr₆₁–Lys₁₀₄) connects the two domains. Cf SP is synthesized as zymogen and the activation site (Lys₁₀₄–Ile₁₀₅) is between the clip domain and the catalytic domain [14, 30].

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1  AATGTCATTCCAAGTTAAATGTGCAATATGGACGATTCGATTCGTCAGCGGCCG
61  GCATGGATTGTCTCTATGTCCTCTACTGTGGCAATTCGACGAGCGCTCTTTTC
1  M D L F Y V S L L A I A A S P A F S
121  CAAAGTACAGTATGCCAATACTATGTCATGGTACATGCGTCACTTGTTCAGCGGAACA
20  Q S T V C Q Y N L H G T C V N L L T G T
181  TOCCACTGTGGAGCAGGGAACCATCTAGTCTTATGTTATGTGGGTTCAGAGGCATGT
40  S H C G A G N H L V L S Y C G F L E A C
241  TGCTATGGAGCCATCAACCGCGCTACGTCTGCTCCACACATGCGCGTCCATGGG
60  C Y G A H Q T A A T S A P T H A P S H G
301  TCGGATCAAGTGTGTTGCGGTTACACCTGAGTAGGGTACCTGGGGTTCCTGCGCTA
80  S G S S G S G S H L S S G T C G L P A V
361  TCTGTAATCAAAAATGTAGGAGGCACACAGCAACCATGGGGAATACCATGGCAA
100  S G N H K I V G G T T A T H G E Y P W Q
421  GTTTCCTTAGATAGGAGGCATCATATGTGGAGGTACCCCTATAGATAATAGTGG
120  V S L R Y G G H H M C G G T L I D N Q W
481  GTTGTCAGCGCGCTCCTGCTTCCAGACACATGAGAGCCACTGGACTGTAGCGGT
140  V V T A A C F Q D T S R S H W T V A V
541  GGTGTCAGGATGCTGGTCAAGTGTACACAGCGAGTTACACTGCCATCAATATATA
160  G V Q D R G H V Y T S Q V H T A I N I I
601  ACACAGCAATATTAGCAATAGTGTACCATAGCATATAGAGCTGATCAATATAGC
180  T H E Y Y D H S R N H N I A L I K L D
661  AACCTGTGACACTAACCACCTTACGTAAAGAGGCACTGTCTTCCTGACCCATGAA
200  K P V D T T T T Y V R I A C L P D P N E
721  GATTGTGACCAACAGTTGTACAGCACTGGATGGGCACTACAGCGGTGGACAA
220  D F D N N V C T A I G W G A T H E G G Q
781  GGTCTGCTTATCTGAGAGAGGTGGACGTACCAATCATTTCAACACACATGTCTCAT
240  G S R Y L R E V D V P I S N N M C H Y
841  TACATGGGTAATCTGTTTACTCATATATATGTCTGGCTTACGTAGGAGTGGAG
260  Y M G N T V Y S S N I C A G F S E G G K
901  GAGCTTGTACAGGAGATTGCGGTGCGCATTAACCTGTAAAGAGAGGTCAATGGAAA
280  D A C Q Q G D G G P L T C K K N G Q W K
961  CTGGCTGTATTAAGTCATGGGATAGGTGTGCTCAAGACACGCGCGGCTCAT
300  L A G I T S W G Y G C A Q R H A P G V Y
1021  ACTGAGTTTGTCTCTCTACTGGATCAAAACCAAGAACTGCCTAAGCTCAT
320  T R V S S F L Y W I Q T I K N S H *
1081  TAATGGTCACTGGCTTCTCTGTATGGGATATAAAGTAATCAGTAGAATCAAAAAA
1141  AAAAAAAAAA

```

Fig. 1 Nucleotide and deduced amino acid sequences of Cf SP gene (DQ280149). The nucleotide and amino acid sequence is numbered on the left. The putative signal peptide is underlined and the cleavage site is indicated by a vertical arrow. The clip domain and SP catalytic domain are double and wave underlined respectively. The conservative catalytic triad (H, D, S) is indicated by black shadow. The asterisk (*) indicates the stop codon. The classical polyadenylation signal is enclosed in a box

Comparisons using BLAST 2.0 at NCBI (<http://www.ncbi.nlm.nih.gov/blast>) revealed, in overall sequence, that the deduced peptide sequence of Cf SP is most similar to the first mollusk serine protease with clip domain CFSP1 (DQ186670) [28], with a 68% identity at the amino acid level. Cf SP is also very similar to *T. tridentatus* proclotting enzyme (AAA30094) [18] and *Ctenocephalides felis* SP8 (AF053921) [33] (27% a.a identity), *C. felis* SP33 (AF053915) [33] and *D. melanogaster* Easter (NM_079638) [27] (26% a.a. identity). In spite of the sequence similarity among different clip domains is low (12–23% a.a identity), six Cys residues corresponding to the three disulfide bonds are strictly conserved (Cys1–Cys5, Cys2–Cys4, Cys3–Cys6; Fig. 2A). Furthermore, clip domains have the similar arrangement of Cys residues to those in beta-defensins of bovine [34] and big defensin, an antibacterial protein isolated from *T. tridentatus* [35]. Therefore, it has been suggested that clip domains may have a fold similar to these antibacterial proteins [14]. And the recombinant of clip domain from *Manduca sexta*

A

	C	C	C	C	CC
Tc def	CAGN	---PDCPSK	---PFRHEY	---VDYYSAVCE	---RYFCG
ENED 3	CPIN	---PDCVPH	---PGRTR	---QIGTF	---GPRINC
Cf SP	CCYN	---LHGCTG	---LGTSH	---GAGNH	---VLESCG
CFSP1	CVAT	---LHGCTG	---YAATVY	---CPLGDS	---FVUSFCG
Tc PCE	CENR	---FTEECG	---CKNLD	---CRILQ	---LQNDYNL
Tc FB	CIAR	---GCLKCS	---KSLID	---CPSLAL	---LTKDS
Ag Sp18d	CSHQ	---CESGCP	---PPYSK	---CKRGNR	---ITVCHSA
Cf SP8	CTRSYD	---GAGGAL	---TQ	---OPSANR	---ILQTTG
Ms HP1	CTLD	---SGKQ	---GCHPH	---PF	---SIQED
Ms HP2	CVD	---NGVT	---CKLPHR	---K	---LSALQ
Cf SP33	CPYK	---GERF	---GCLIS	---C	---VLGGG
Dm snake	CPRSF	---GCRS	---CGLAYQ	---G	---LHIREY
Ms PAP1	CTTD	---QVVD	---SNCS	---SYE	---CPOLLS
Ms PAP2	CTLD	---NNDC	---CKRSLQ	---	---QVASKI
Ms PAP3	CTTD	---NNMG	---CKRSLY	---	---SEELK
Bm PAP1	CTTD	---MLN	---GCHPH	---	---QADLAL
Ag Sp14a	CTTD	---DHRD	---GCHPH	---	---QPSRD
Ag Sp14d1	CYND	---VCEA	---GCLFPE	---	---QCPVD
Ag Sp14d2	CTTD	---DCKV	---GCHPH	---	---CLSDH
Hd PPAF	CTTD	---NGEN	---GCHPH	---	---CKITVD
S1 PPAE1	CTTD	---TCSR	---GCHPH	---	---QCPYV
S1 PPAE3	CTTD	---SGAN	---GCHPH	---	---QVLDL
Dm easter	CTTD	---NRRR	---GCHPH	---	---CKTYG

B

Cf SP	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
CFSP1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Tc PCE	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Tc FB	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ag Sp18d	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Cf SP8	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ms HP1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ms HP2	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Cf SP33	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Dm snake	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ms PAP1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ms PAP2	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ms PAP3	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Bm PAP1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ag Sp14a	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ag Sp14d1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Ag Sp14d2	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Hd PPAF	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
S1 PPAE1	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
S1 PPAE3	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ
Dm easter	RIUGCTTATGCGPWSV	---P	GGG	---HHM	---CGG	---TIDN	---QVMTAA	---HCFQ

Cf SP	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
CFSP1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Tc PCE	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Tc FB	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ag Sp18d	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Cf SP8	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ms HP1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ms HP2	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Cf SP33	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Dm snake	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ms PAP1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ms PAP2	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ms PAP3	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Bm PAP1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ag Sp14a	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ag Sp14d1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Ag Sp14d2	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Hd PPAF	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
S1 PPAE1	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
S1 PPAE3	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---
Dm easter	YLPEDDVPIISNNHCHYHGN	---T	---	---	---	---	---	---

Cf SP	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
CFSP1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Tc PCE	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Tc FB	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ag Sp18d	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Cf SP8	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ms HP1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ms HP2	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Cf SP33	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Dm snake	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ms PAP1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ms PAP2	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ms PAP3	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Bm PAP1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ag Sp14a	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ag Sp14d1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Ag Sp14d2	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Hd PPAF	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
S1 PPAE1	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
S1 PPAE3	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---
Dm easter	DTSPSHMTVA	---QVD	GHV	---	---	---	---	---

Cf SP	KNC	---	---	---	---	---	---	---
CFSP1	KNC	---	---	---	---	---	---	---
Tc PCE	KNC	---	---	---	---	---	---	---
Tc FB	KNC	---	---	---	---	---	---	---
Ag Sp18d	KNC	---	---	---	---	---	---	---
Cf SP8	KNC	---	---	---	---	---	---	---
Ms HP1	KNC	---	---	---	---	---	---	---
Ms HP2	KNC	---	---	---	---	---	---	---
Cf SP33	KNC	---	---	---	---	---	---	---
Dm snake	KNC	---	---	---	---	---	---	---
Ms PAP1	KNC	---	---	---	---	---	---	---
Ms PAP2	KNC	---	---	---	---	---	---	---
Ms PAP3	KNC	---	---	---	---	---	---	---
Bm PAP1	KNC	---	---	---	---	---	---	---
Ag Sp14a	KNC	---	---	---	---	---	---	---
Ag Sp14d1	KNC	---	---	---	---	---	---	---
Ag Sp14d2	KNC	---	---	---	---	---	---	---
Hd PPAF	KNC	---	---	---	---	---	---	---
S1 PPAE1	KNC	---	---	---	---	---	---	---
S1 PPAE3	KNC	---	---	---	---	---	---	---
Dm easter	KNC	---	---	---	---	---	---	---

Cf SP	IALEMLDKP	---D	---	---	---	---	---	---
CFSP1	IALEMLDKP	---D	---	---	---	---	---	---
Tc PCE	IALEMLDKP	---D	---	---	---	---	---	---
Tc FB	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp18d	IALEMLDKP	---D	---	---	---	---	---	---
Cf SP8	IALEMLDKP	---D	---	---	---	---	---	---
Ms HP1	IALEMLDKP	---D	---	---	---	---	---	---
Ms HP2	IALEMLDKP	---D	---	---	---	---	---	---
Cf SP33	IALEMLDKP	---D	---	---	---	---	---	---
Dm snake	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP1	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP2	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP3	IALEMLDKP	---D	---	---	---	---	---	---
Bm PAP1	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14a	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14d1	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14d2	IALEMLDKP	---D	---	---	---	---	---	---
Hd PPAF	IALEMLDKP	---D	---	---	---	---	---	---
S1 PPAE1	IALEMLDKP	---D	---	---	---	---	---	---
S1 PPAE3	IALEMLDKP	---D	---	---	---	---	---	---
Dm easter	IALEMLDKP	---D	---	---	---	---	---	---

Cf SP	IALEMLDKP	---D	---	---	---	---	---	---
CFSP1	IALEMLDKP	---D	---	---	---	---	---	---
Tc PCE	IALEMLDKP	---D	---	---	---	---	---	---
Tc FB	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp18d	IALEMLDKP	---D	---	---	---	---	---	---
Cf SP8	IALEMLDKP	---D	---	---	---	---	---	---
Ms HP1	IALEMLDKP	---D	---	---	---	---	---	---
Ms HP2	IALEMLDKP	---D	---	---	---	---	---	---
Cf SP33	IALEMLDKP	---D	---	---	---	---	---	---
Dm snake	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP1	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP2	IALEMLDKP	---D	---	---	---	---	---	---
Ms PAP3	IALEMLDKP	---D	---	---	---	---	---	---
Bm PAP1	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14a	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14d1	IALEMLDKP	---D	---	---	---	---	---	---
Ag Sp14d2	IALEMLDKP	---D	---	---	---	---	---	---
Hd PPAF	IALEMLDKP	---D	---	---	---	---	---	---
S1 PPAE1	IALEMLDKP	---D	---	---	---	---	---	---
S1 PPAE3	IALEMLDKP	---D	---	---	---	---	---	---
Dm easter	IALEMLDKP	---D	---	---	---	---	---	---

Fig. 2 (A) Sequence alignment of clip domains from mollusk and arthropod serine proteases, including *C. farreri* serine proteases: Cf SP (DQ280149) and CFSP1 (DQ186670); *T. tridentatus* proclotting enzyme: Tt PCE (AAA30094) and Clotting factor B: Tt FB (BAA03528); *Anopheles gambiae* serine proteases: Ag Sp14a (AF117748), Ag Sp14d1 (AY341209), Ag Sp14d2 (AF117749) and Ag Sp18d (AF117750); *C. felis* serine proteases: Cf SP8 (AF053921) and Cf SP33 (AF053915); *M. sexta* serine proteases: Ms HP1 (AF017663) and Ms HP2 (AF017664); *M. sexta* prophenoloxidase-activating proteinases: Ms PAP1 (AAX18636), Ms PAP2 (AAL76085) and Ms PAP3 (AAX18637); *D. melanogaster* serine proteases: Dm Easter (NM_079638) and Dm snake (NM_079614); *B. mori* prophenoloxidase activating enzyme: Bm PPAE1 (AB009670); *H. diomphalia* prophenoloxidase activating factor: Hd PPAF (AJ400903); *Spodoptera litura* prophenol oxidase activating enzymes: Sl PPAE1 (AY677081) and Sl PPAE3 (AY677082). Six Cys residues that form three disulfide bonds (Cys-1–Cys-5, Cys-2–Cys-4 and Cys-3–Cys-6) are conserved in all of the sequences. Above the clip domains are the aligned sequences or regions of big defensin from *T. tridentatus* (E11206) and bovine neutrophil β -defensin3 (AF016396), to demonstrate a similarity in the pattern of Cys residues. (B) Sequence alignment of the carboxyl-terminal catalytic domains of serine proteases from mollusk and arthropod serine proteases with clip domain. Identical amino acids are in white letters with black background, and gray background indicates high levels of amino acid similarity

cuticular proPO-activating proteinase (PAP) has been observed to be toxic to the *Escherichia coli* host [14, 27]. The catalytic domains in these proteases have a higher similarity of 34–82% (a.a. identity) (Fig. 2B.). In addition, the catalytic domain of Cf SP has also similar sequence identities to several mammalian protease family members that are involved in immune and wound responses, such as plasma kallikrein B1 (NP_000883) [36] and plasminogen (NP_032903) [37].

A phylogenetic tree was constructed using the overall amino acid sequences alignment under the neighbor-joining criterion (Fig. 3.). In all serine proteases with

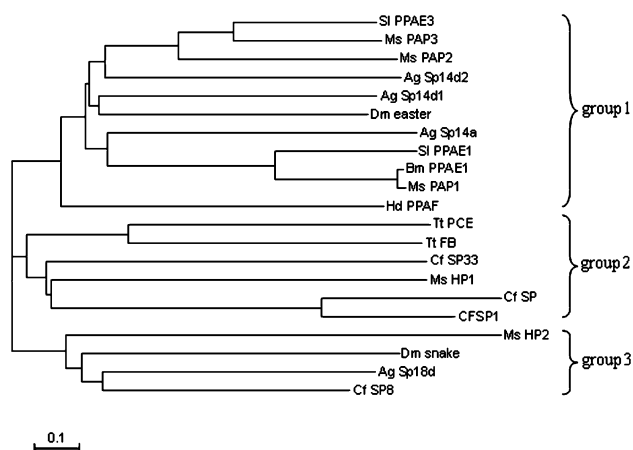


Fig. 3 A phylogenetic tree was constructed using the overall amino acid sequences alignment under the neighbor-joining criterion

clip domain, known as putative proPO-activating proteinases (PAPs) or proPO-activating enzymes (PPAEs) formed a cluster (group 1), and the mollusk serine proteases Cf SP and CFSP1, *M. sexta* and *C. felis* immunity-related serine proteases HP1 (AF017663) and SP33, *T. tridentatus* proclotting enzyme and coagulation factor B precursor formed the group 2. Other development-related serine proteases with clip domain formed another cluster (group 3) in the phylogenetic analysis. The cluster result of serine proteases with clip domain may demonstrate the functional similarity of the same group [14].

Northern blot analysis using DIG-labeled Cf SP probe was performed to verify the molecular size of Cf SP mRNA and the tissue distribution (Fig. 4). An approximately 1.2-kb transcript of Cf SP gene from *C. farreri* could be detected in hemocytes, which was strongly expressed, and in mantle margin, which was weakly, but could not be detected in gonad, digestive gland, heart, intestine, gill and adductor muscle. In insect and crustacean, several immunity-related serine protease with clip domain were mainly expressed in hemocytes and/or fat body, such as the prophenoloxidase (proPO) system related serine proteases of tobacco hornworm *M. sexta* PPA 1, PPAF 2, and PPAF 3 [27, 38, 39], the Masquerade-like serine proteases of crayfish *Pacifastacus leniusculus* [40], and the hemolymph clotting system related serine proteases of horseshoe crabs *T. tridentatus* proclotting enzyme and factor B [14, 18, 19]. Hemocytes and fat body were two major expression sites of immune-related proteins and release biodefense genes or activate the host's biodefense mechanisms [41]. These facts suggest that Cf SP might be an immune-related protein and was synthesized in hemolymph.

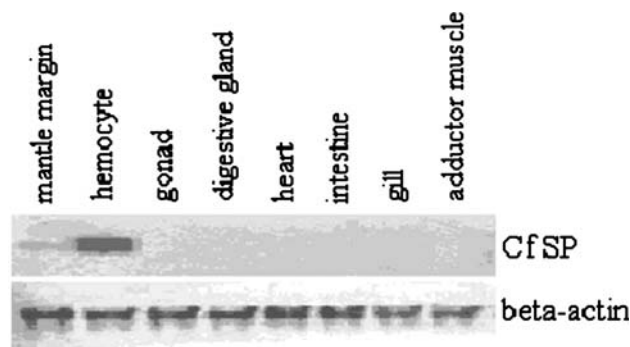


Fig. 4 Northern blotting analysis of Cf SP transcript expression distribution and relative abundance in hemocyte, mantle margin, gonad, digestive gland, heart, intestine, gill and adductor muscle. To detect the weak transcript of Cf SP gene, the magnitude of RNA and probe for Cf SP was three-fold than that of beta-actin. Northern blotting using a Cf SP revealed the presence of an approximately 1.2-kb transcript of Cf SP gene

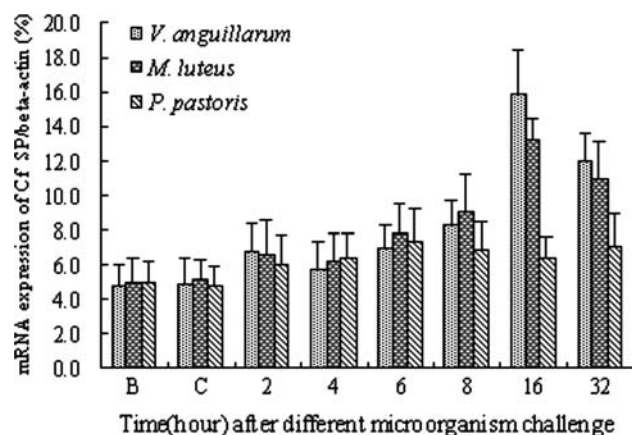


Fig. 5 mRNA expression of Cf SP in hemolymph at different time points after microorganism challenge. “mRNA expression of Cf SP/beta-actin (%)” indicates the ratio obtained from the density of the band of Cf SP mRNA expression standardized with that of the beta-actin mRNA expression level. B: Blank group (untreated scallops). C: control group (scallops received an injection of 50 ml PBS)

The temporal expression of the Cf SP gene by microorganism challenge was measured by semi-quantitative RT-PCR. At 2, 4, 6 and 8 h post-injection, there was no significant difference in Cf SP gene expression among the control, blank and challenged samples (Fig. 5). At 16 h after injection, the expression of the Cf SP gene was up-regulated in the *V. anguillarum* and the *M. luteus* challenged groups, increasing, respectively 3.2-fold and 2.6-fold, compared to the blank, and then dropped progressively. Analysis of variance indicated that the Cf SP gene expression at 16 h post-*V. anguillarum*-challenge and post-*M. luteus*-challenge were significantly higher ($F = 9.277$, $P < 0.05$; $F = 6.764$, $P < 0.05$, respectively) than that at other time points. Under the *P. pastoris* challenged group, there was no significant difference at all time points ($F = 1.697$, $P > 0.05$). Similar to observations of serine proteases with clip domain have been reported in arthropods, such as the immune responsive factor D-like serine proteinase from *Dermacentor variabilis* [42], the *M. sexta* and *C. felis* immunity-related serine proteases HP1 [43] and SP33 [33]. These results indicated that Cf SP was induced by Gram-negative and Gram-positive bacteria challenge respectively, but was not done by fungal challenge.

Acknowledgements The authors would like to thank all laboratory members for technical advice and helpful discussions. This research was supported by 863 High Technology Project (No. 2002AA626020) from the Chinese Ministry of Science and Technology, the grant (No. 40276045 and 30230280) from NSFC to Dr. Linsheng Song and Prof. Jianhai Xiang, and the grant (No.06SB011014) from South China Sea Institute of Oceanography, Chinese Academy of Sciences to Dr. Ling Zhu.

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