Mol Biol Rep (2008) 35:257–264
DOI 10.1007/s11033-007-9078-2

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

Ling Zhu · Linsheng Song · Yuze Mao · Jiangmin Zhao · Chenghua Li · Wei Xu

Received: 17 January 2007 / Accepted: 9 April 2007 / Published online: 6 May 2007
© Springer Science+Business Media B.V. 2007

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

L. Zhu (✉) · L. Song (✉) · J. Zhao · C. Li · W. Xu
Institute of Oceanology, Chinese Academy of Sciences, 7 Nanhai Rd, Qingdao 266071, P.R. China
e-mail: llingzhu@163.com

L. Song
e-mail: lshsong@ms.qdio.ac.cn

L. Zhu
Key Laboratory of Tropical Marine Environmental Dynamics (LED), South China Sea Institute of Oceanography, Chinese Academy of Sciences, Guangzhou 510301, P.R. China

Y. Mao
Mariculture Ecology Division, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, P.R. China

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

Seikagaku Corporation Exhibit 2088 - Page 1 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

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 *Vibro anguillarum*, *Micrococcus luteus* and *Pichia pastoris*, respectively. They were collected by centrifugation (10,000*g*, 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 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 1,000*g*, 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 EST_{344} 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.

Seikagaku Corporation Exhibit 2088 - Page 2 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

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} $MgCl_2$, 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 $MgCl_2$ (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

Seikagaku Corporation Exhibit 2088 - Page 3 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

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 conductive 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 EST_{344} 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 Ser_{19} and Gln_{20}. 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 (Cys_{24}–Cys_{60}), which stabilized by three disulfide bonds formed between six conserved Cys residues, a C-terminal typical trypsinogen-like catalytic domain (Ile_{105}–Ile_{329}) with the conserved His–Asp–Ser catalytic triad, and a linking sequence (Tyr_{61}–Lys_{104}) connects the two domains. Cf SP is synthesized as zymogen and the activation site (Lys_{104}–Ile_{105}) is between the clip domain and the catalytic domain [14, 30].

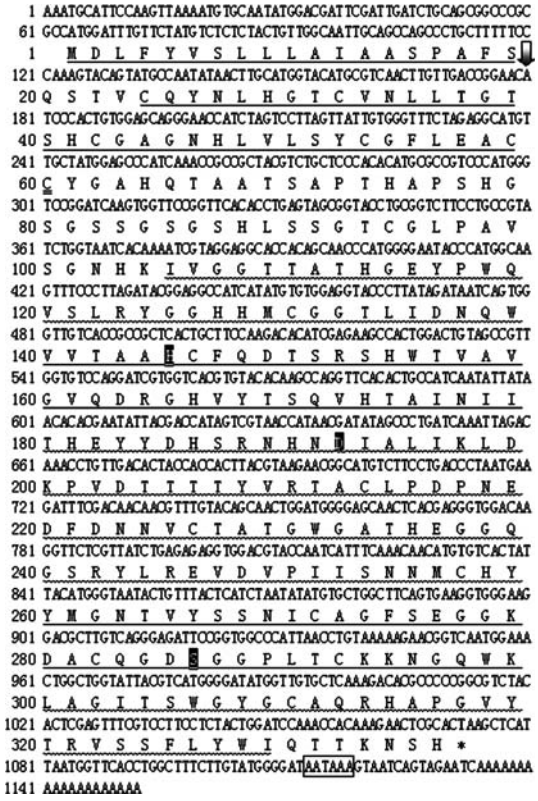
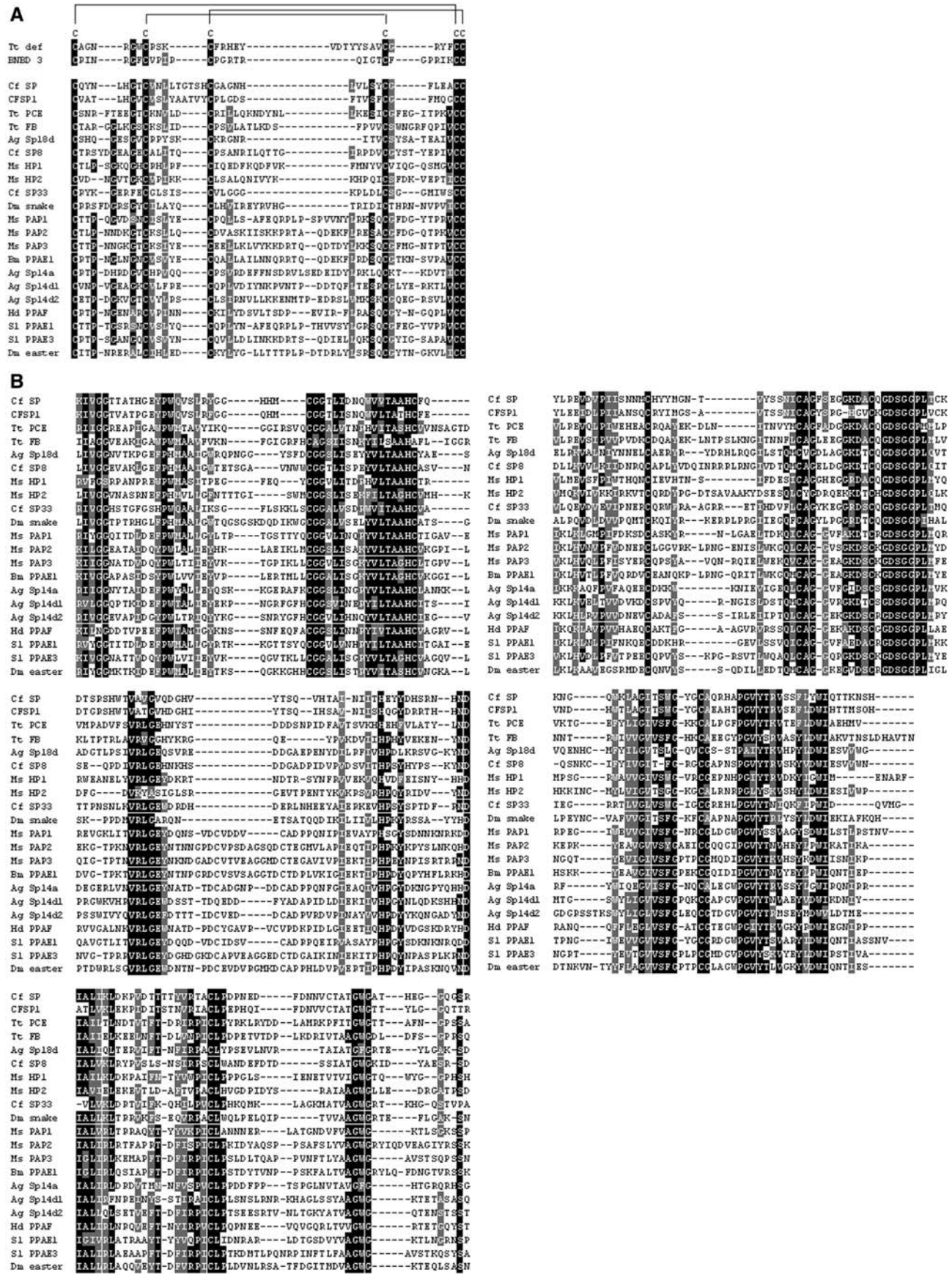


Fig. 1 Nucleotide and deduced amino acid sequences of Cf SP gene (DQ280149). The nucleotide and amino acid sequence is numbered on the left. The putative signal peptide is underlined and the cleavage site is indicated by a vertical arrow. The clip domain and SP catalytic domain are double and wave underlined 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*

Seikagaku Corporation Exhibit 2088 - Page 4 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023



Seikagaku Corporation Exhibit 2088 - Page 5 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

◀**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 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.

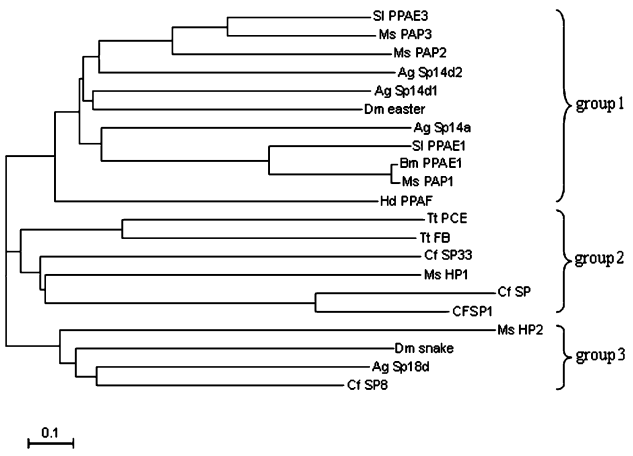


Fig. 3 A phylogenetic tree was constructed using the overall amino acid sequences alignment under the neighbor-joining criterion

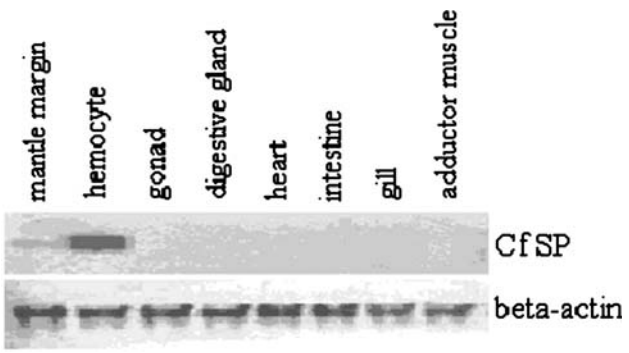


Fig. 4 Northern blotting analysis of Cf SP transcript expression distribution and relative abundance in hemocyte, mantle margin, gonad, digestive gland, heart, intestine, gill and adductor muscle. To detect the weak transcript of Cf SP gene, the magnitude of RNA and probe for Cf SP was three-fold than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

Seikagaku Corporation Exhibit 2088 - Page 6 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

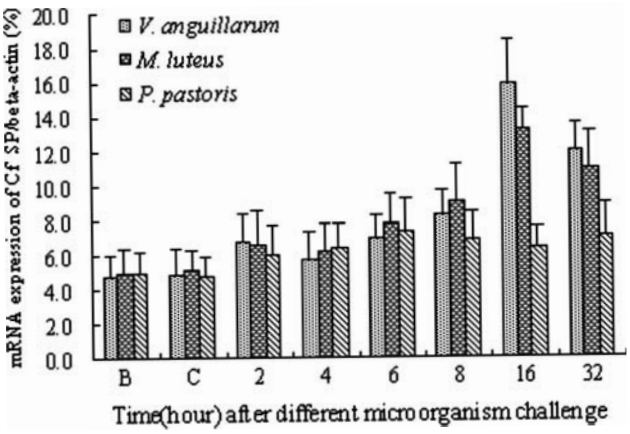


Fig. 5 mRNA expression of Cf SP in hemolymph at different time points after microorganism challenge. ''mRNA expression of Cf SP/beta-actin (%)'' indicates the ratio obtained from the density of the band of Cf SP mRNA expression standardized with that of the beta-actin mRNA expression level. B: Blank 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.

References

1. Guo X, Ford SE, Zhang F (1999) Molluscan aquaculture in China. J Shellfish Res 18:19–31
2. Wang Y, Xiang J (1999) Studies on causation of the mass mortality of *Chlamys farreri*. Oceanol Limnol Sinica 30:770–774, (in Chinese with English abstract)
3. He G, Li Y, Song W, Wang C, Huang J, Wang X (2003) The relationship between pathogenic infection status and mortality of the scallop *Chlamys farreri*. J Fish China 27:273–277
4. Hoffmann JA, Reichhart JM (2002) *Drosophila* innate immunity, an evolutionary perspective. Nat Immunol 3:121–126
5. Hoffmann JA (2003) The immune response of *Drosophila*. Nature 426:33–38
6. Seo J, Crawford JM, Stone KL, Noga EJ (2005) Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*. Biochem Biophys Res Commun 338:1998–2004
7. Zhao J, Song L, Li C, Zou H, Ni D, Wang W, Xu W (2007) Molecular cloning of an invertebrate goose-type lysozyme gene from *Chlamys farreri*, and lytic activity of the recombinant protein. Mol Immunol 44:1198–1208
8. Medzhitov R, Janeway CA Jr (1997) Innate immunity, impact on the adaptive immune response. Curr Opin Immunol 9:4–9
9. Medzhitov R, Janeway CA Jr (2002) Decoding the patterns of self and nonself by the innate immune system. Science 296:298–300
10. Michel T, Reichhart JM, Hoffmann JA, Royet J (2001) *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature 414:756–759
11. Christophides GK, Zdobnov E, Barillas-Mury C, Birney E, Blandin S, Blass C, Brey PT, Collins FH, Danielli A, Dimopoulos G, Hetru C, Hoa NT, Hoffmann JA, Kanzok SM, Letunic I, Levashina EA, Loukeris TG, Lycett G, Meister S, Michel K, Moita LF, Muller HM, Osta MA, Paskewitz SM, Reichhart JM, Rzhetsky A, Troxler L, Vernick KD, Vlachou D, Volz J, von Mering C, Xu J, Zheng L, Bork P, Kafatos FC (2002) Immunity-related genes and gene families in *Anopheles gambiae*. Science 298:159–165
12. Dimopoulos G, Muller HM, Levashina EA, Kafatos FC (2001) Innate immune defense against malaria infection in the mosquito. Curr Opin Immunol 13:79–88
13. Osta MA, Christophides GK, Vlachou D, Kafatos FC (2004) Innate immunity in the malaria vector *Anopheles gambiae*: comparative and functional genomics. J Exp Biol 207:2551–2563
14. Jiang H, Kanost MR (2000) The clip-domain family of serine proteinases in arthropods. Insect Biochem Mol Biol 30:95–105
15. Kanost MR, Jiang H, Wang Y, Yu XQ, Ma C, Zhu Y (2001) Hemolymph proteinases in immune responses of *Manduca sexta*. Adv Exp Med Biol 484:319–328
16. De Gregorio E, Spellman PT, Rubin GM, Lemaitre B (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. Proc Natl Acad Sci USA 98:12590–12595
17. Ligoxygakis P, Pelte N, Hoffmann JA, Reichhart JM (2002) Activation of *Drosophila* Toll during fungal infection by a blood serine protease. Science 297:114–116
18. Muta T, Hashimoto R, Miyata T, Nishimura H, Toh Y, Iwanaga S (1990) Proclotting enzyme from horseshoe crab hemocytes. cDNA cloning, disulfide locations, and subcellular localisation. J Biol Chem 265:22426–22433
19. Muta T, Oda T, Iwanaga S (1993) Horseshoe crab coagulation factor B. A unique serine protease zymogen activated by cleavage of an Ile-Ile bond. J Biol Chem 268:21384–21388

Seikagaku Corporation Exhibit 2088 - Page 7 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023

20. Dohke K (1973a) Studies on prophenoloxidase-activating enzyme from cuticle of the silkworm *Bombyx mori*. I. Activation reaction by the enzyme. Arch Biochem Biophys 157:203–209
21. Dohke K (1973b) Studies on prephenoloxidase-activating enzyme from cuticle of the silkworm *Bombyx mori*. II. Purification and characterization of the enzyme. Arch Biochem Biophys 157:210–221
22. Aspán A, Hall M, Söderhäll K (1990) The effect of endogenous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase from crayfish haemocytes. Insect Biochem 20:485–492
23. Aspán A, Söderhäll K (1991) Purification of prophenoloxidase from crayfish blood cells and its activation by an endogenous serine proteinase. Insect Biochem 21:363–373
24. Chosa N, Fukumitsu T, Fujimoto K, Ohnishi E (1997) Activation of prophenoloxidase A1 by an activating enzyme in *Drosophila melanogaster*. Insect Biochem Mol Biol 27:61–68
25. Lee SY, Kwon TH, Hyun JH, Choi JS, Kawabata SI, Iwanaga S, Lee BL (1998a) In vitro activation of prophenoloxidase by two kinds of prophenoloxidase activating factors isolated from hemolymph of coleopteran, *Holotrichia diomphalia* larvae. Eur J Biochem 254:50–57
26. Lee SY, Cho MY, Hyun JH, Lee KM, Homma KI, Natori S, Kawabata SI, Iwanaga S, Lee BL (1998b) Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3–glucan in coleopteran insect, *Holotrichia diomphalia* larvae. Eur J Biochem 257:615–621
27. Jiang HB, Wang Y, Kanost MR (1998) Pro-phenol oxidase activating proteinase from an insect, Manduca Sexta: A bacteria-inducible protein similar to Drosophila easter. Proc Natl Acad Sci 95:12220–12225
28. Zhu L, Song L, Zhao J, Li Ch, Chang Y (2006) Molecular cloning, characterization and expression of a serine proteinase with clip-domain homologue from scallop *Chlamys farreri*. Fish Shellfish Immunol doi:10.1016/j.fsi.2006.08.002
29. Jacquinet E, Rao NV, Rao GV, Hoidal JR (2000) Cloning, genomic organization, chromosomal assignment and expression of a novel mosaic serine protease: epitheliasin. FEBS Lett 468:93–100
30. Ross J, Jiang H, Kanost MR, Wang Y (2003) Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationship. Gene 304:117–131
31. Kumar SK, Tamura K, Jakobsen IB, Nei M (2000) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17:1244–1245
32. Kawabata S, Muta T, Iwanaga S (1996) Clotting cascade and defense molecules found in the hemolymph of the horseshoe crab. In: Söderhäll K, Iwanaga S, Vasta G (eds) New directions in invertebrate immunology. Fair Haven, NJ, SOS publication, pp 255–283
33. Gaines PJ, Sampson CM, Rushlow KE, Stiegler GL (1999) Cloning of a family of serine protease genes from the cat flea *Ctenocephalides felis*. Insect Mol Biol 8:11–22
34. Yount NY, Yuan J, Tarver A, Castro T, Diamond G, Tran PA, Levy JN, McCullough C, Cullor JS, Bevins CL, Selsted ME (1999) Cloning and expression of bovine neutrophil β-defensins: biosynthetic profile during neutrophilic maturation and localization of mature peptide to novel cytoplasmic dense granules. J Biol Chem 274:26249–26258
35. Iwanaga S, Kawabata S, Muta T (1998) New types of clotting factors and defense molecules in horseshoe crab hemolymph: their structures and functions. J Biochem 123:1–15
36. Dulinski R, Suder P, Guevara-Lora I, Rapala-Kozik M, Potempa J, Silberring J, Imamura T, Travis J, Kozik A (2003) Attenuated kinin release from human neutrophil-elastase pretreated kininogens by tissue and plasma kallikreins. Biol Chem 384:929–937
37. Bannach FG, Gutierrez A, Fowler BJ, Bugge TH, Degen JL, Parmer RJ, Miles LA (2002) Localization of regulatory elements mediating constitutive and cytokine-stimulated plasminogen gene expression. J Biol Chem 277:38579–38588
38. Dohke K (1973) Studies on prephenoloxidase-activating enzyme from cuticle of the silkworm *Bombyx mori*. II. Purification and characterization of the enzyme. Arch Biochem Biophys 157:210–221
39. Jiang H, Wang Y, Yu X-Q, Kanost MR (2003) Prophenoloxidase-activating proteinase-2 (PAP-2) from hemolymph of *Manduca sexta*: a bacteria-inducible serine proteinase containing two clip domains. J Biol Chem 278:3552–3561
40. Kopacek P, Grubnoffer L, Söderhäll K (1993) Isolation and. characterization of a hemagglutinin with affinity for lipoplysaccharides from. plasma of the crayfish *Pacifastacus leniusculus*. Dev Comp Immunol 17:407–418
41. Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. Insect Biochem Mol Biol 32:1295–1309
42. Simser JA, Mulenga A, Macaluso KR, Azad AF (2004) An immune responsive factor D-like serine proteinase homologue identified from the American dog tick, Dermacentor variabilis. Insect Mol Biol 13:25–35
43. Jiang H, Wang Y, Kanost MR (1999) Four serine proteinases expressed in *Manduca sexta* haemocytes. Insect Mol Biol 8:39–53

View publication stats

Seikagaku Corporation Exhibit 2088 - Page 8 of 8
Charles River Laboratories, Inc. v. Seikagaku Corporation
PGR2025-00023