

Cloning and characterisation of a serine proteinase from the haemocytes of mud crab *Scylla serrata*

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Abstract

A serine proteinase (SP) cDNA was cloned from the haemocytes of mud crab *Scylla serrata* using oligonucleotide primers and RT–PCR. Both 3′- and 5′-regions were isolated by rapid amplification of cDNA end (RACE) method. Analysis of the nucleotide sequence revealed that the cDNA clone has an open reading frame of 1131 bp encoding a protein of 376 amino acids. The calculated molecular mass of the SP mature protein is 39.54 kDa with an estimated *pI* of 5.37. The C-terminal half of *S. serrata* SP is composed of a trypsin-like domain, with a sequence similar to that of other invertebrate and vertebrate SP domain. The typical catalytic triad of SP required for functional activity (His150, Asp217 and Ser331) was conserved in the polypeptide sequence. Sequence comparison showed that SP deduced amino acid has an overall similarity of 55%, 51% and 50% to SP deduced amino acid from spiny lobster *Panulirus argus*, horseshoe crab *Tachypleus tridentatus* and crayfish *Pacifastaus leniusculus*, respectively. The SP was strongly expressed in haemocytes, but was weakly expressed in heart, eyestalk and antennules. The SP transcript decreased significantly for the *S. serrata* following 3 days exposure to pH 9.5. However, the SP transcript increased significantly 24 h post-zymosan injection.

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Keywords: Mud crab; *Scylla serrata*; Serine proteinase; Immunity; Clip domain; pH; Zymosan injection

1. Introduction

The mud crab *Scylla serrata* has been cultured in Taiwan and south east coast of China for at least 100 years and throughout the Indo-Pacific regions for more than 30 years [1]. Several viral diseases such as reolike virus, rhabdovirus A, picornalike virus, herpeslike virus (HLV), white spot syndrome virus (WSSV) and vibriosis caused by *Vibrio parahaemolyticus* have been reported to infect crabs [2,3]. These pathogenic viruses and bacteria infected the haemocytes and epithelial cells, and are associated with mass mortalities during disease outbreaks. It is known that invertebrates lack a true adaptive immune system and rely instead on various innate immune responses against invading pathogens. Research on the innate immune system will provide new insights into the management and control of infectious diseases.

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The prophenoloxidase (proPO) is activated to its active form phenoloxidase (PO) through a limited proteolysis by endogenous serine proteinase (SP) that leads to melanisation through a complex enzymatic cascade [4,5]. Proteolytic activation of proPO is an integral part of the immune system against pathogen and parasitic infections. The active form of proPO, phenoloxidase (PO), is produced by serine proteinase, a so-called prophenoloxidase-activating factor (PPAF) [5,6]. Various insect serine proteinases (SPs) have been reported to be involved in the activation of the proPO system [7,8] similar to that of trypsin-like SP in crayfish *Pacifastacus leniusculus* [9,38]. Serine proteinase homologues (SPHs) are very similar in amino acid sequence to SPs except for one or more substitutions within the catalytic triad that will render them inactive, without proteinase activity [10].

Serine proteinases are involved in many important processes. For example, SP is involved in antimicrobial response in horseshoe crab *Tachypleus tridentatus* [11], pattern recognition in crayfish *P. leniusculus* [12], somatic muscle attachment in fruit fly *Drosophila* embryos [8] and immune response in African malaria mosquito *Anopheles gambiae* [13]. The SPs and SPHs have been cloned and characterised in domestic silkworm *Bombyx mori* [24], crayfish *P. leniusculus* [12,14], coleopteran *Holotrichia diomphalia* [7,15], kuruma shrimp *Marsupenaeus japonicus* [16], white shrimp *Litopenaeus vannamei* [17], horseshoe crab *T. tridentatus* [18], spiny lobster *Panulirus argus* [19], and centipede *Scolopendra subspinipes mulilans* [20].

The aim of the present study was (1) to present the nucleotide sequence of serine proteinase from the haemocytes of *S. serrata*, and compare its sequence with other serine proteinases, (2) to evaluate this SP expression when *S. serrata* under high pH stress and when injected with zymosan, and (3) to examine the expression of SP from various tissues of *S. serrata*.

2. Materials and methods

2.1. Collection and maintenance of mud crab *S. serrata*

S. serrata (65–90 mm in width) collected from a farm in Ilan, Taiwan, were acclimatised in plastic turf containing 35‰ seawater for 3 days. They were fed daily with fish or shrimp meat at 10% of body weight.

2.2. RNA isolation and cDNA synthesis by RT–PCR

Haemolymph (50 ml) was withdrawn by inserting a syringe into the sinus at the base of right chelate leg into a 125-ml polyethylene tube containing 50 ml of pre-cooled (4 °C) anticoagulant (10% trisodium citrate) [21]. The diluted haemolymph was centrifuged at $500 \times g$ at 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation. Total RNA was isolated and further purified using the guanidinium thiocyanate method described by Chomczynski and Sacchi [22]. First-strand cDNA synthesis in RT (reverse transcription) was performed by using Superscript™ III RNase H[−] reverse transcriptase (Invitrogen, Carlsbad, CA, USA) to transcribe poly(A)⁺ RNA with oligo-d(T)₁₈ as the primers. Reaction conditions recommended by the manufacturer were followed.

2.3. Degenerate primer design and strategy of SP cDNA cloning

Full-length SP cDNA of *S. serrata* was obtained by the procedures of reverse transcription–polymerase chain reaction (RT–PCR) and rapid amplification of cDNA ends (RACE) method. Multiple alignments and phylogenetic comparisons of SP amino acid sequences of *S. serrata* with other decapod crustaceans were performed. Degenerate primers were designed based on the highly conserved nucleotide sequences of SP of freshwater crayfish *P. leniusculus* [12], a coleopteran *H. diomphalia* [7], tobacco hornworm *Manduca sexta* [23] and domestic silkworm *B. mori* [24] in the GenBank database [25] using the CLUSTAL program [26]. The schematic representations of the degenerate and gene-specific primers used to obtain SP from *S. serrata* are shown in Fig. 1. The primer pairs used for *S. serrata* SP cDNA clone are shown in Table 1. The primers of SP 1F and SP 1R, SP 2F and SP 2R were used to amplify the partial cDNA of SP and cDNA from *S. serrata* were used as templates for PCR (GeneAmp PCR system 2700, Applied Biosystems, Foster, CA, USA). The PCR reaction buffer was 50 mM Tris–HCl buffer (pH 9) containing 50 mM KCl, 1% Triton X-100, 2.5 mM MgCl₂, 2.5 U Taq polymerase, 0.25 mM dNTPs, and 10 μM of each primer. The PCR reactions were performed as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min, followed by a 7-min extension at 72 °C and cooling to 4 °C. Briefly, the first fragment of partial SP

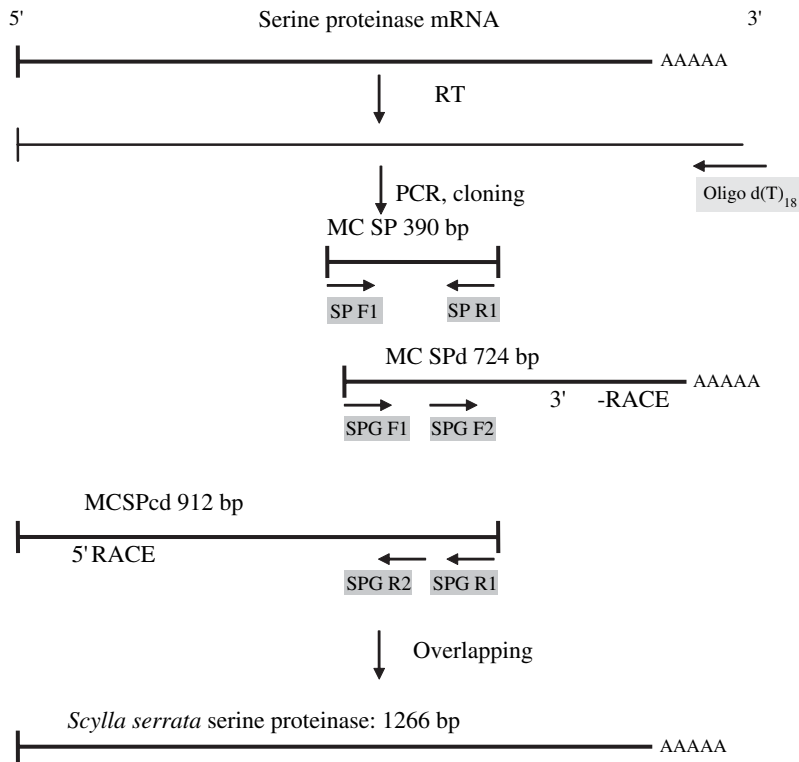


Fig. 1. Schematic diagram showing the relative positions of forward and reverse primers of *S. serrata* SP. Primers SP F1 and SP R1 are degenerate primers. Primers SPG F1, SPG R1 SPG F2 and SPG R2 are gene-specific primers.

cDNA was obtained using primer SP 1F and SP 1R, then 5'- and 3'-RACE were performed to make a full-length SP gene of *S. serrata* using the gene-specific forward primer SPG 1F and reverse primer SPG 1R (Table 1).

2.4. PCR and 5'- and 3'-RACE

Both 3'- and 5'-RACE were performed to generate the full-length of *S. serrata* SP gene using the Gene Racer™ Kit (Catalog No. Li502-02, Invitrogen). Five micrograms of total RNA were used to synthesise the first-strand cDNA in RT using Superscript™ III (Invitrogen). The PCR reaction mixture was prepared following the manufacturer's instructions. Random primers and gene-specific primers from the cloned SP fragment were used. The Primer SPG 1R was used for the 5'-RACE reaction and SPG 1F was used for the 3'-RACE reaction. For 3'-RACE PCR, first

Table 1
Specific primers and degenerate primers used in this study

Primer name	Primer sequence (5' to 3')
SP 1F	CTGARATCGASGASWATCGTTGTGGGWTGG
SP 1R	TACGTTKKTGCACTGCRGCWCACTGTGT
SP 2F	ATCTTTACCCCTGTCAACCCGCCAGCGCA
SP 2R	CATGAGGCGGACCGCCGGAGTCACCTTGCC
SPG 1F	GGTTGTGGGAGTTGCCGACCACAACATGCA
SPG 1R	CAGGTGTCCTTACCACCTTCCTCAAGACCA
SPG 2F	GACAGTGATATCGCGCTAATTACGCTGTCA
SPG 2R	AGTCACCTTGGCAGGTGTCCTTACCACCTT
β-Actin F	TAGGTGGTCTCGTGGATGCC
β-Actin R	GAGACCTCAACACCCCGC

R: A/G; W: A/T; S: G/C; K: G/T.

step five cycles with 92 °C for 1 min, 72 °C for 1 min, and second step five cycles with 92 °C for 1 min, 70 °C for 1 min, third step 30 cycles with 92 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and final extension step 72 °C for 7 min. 5'-RACE was performed using Gene Racer RNA Oligo to decapped the RNA and RT-PCR based on the manufacturer's instructions in the Gene RacerTM Kit (Invitrogen). The PCR conditions were the same as that described above.

2.5. Cloning, sequencing and sequence analysis

PCR products containing a single band were purified using a PCR purification Kit (Promega Corp., Madison, WI, USA) to remove excess primers and dNTPs. The PCR products were ligated into PCR^R II TOPO^R vector and the transformation of JM109 high-efficiency competent *Escherichia coli* cells were performed using the PCR^R II TOPO^R Vector Kit (Invitrogen). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Individual colonies grown on LB (Luria Bertani) agar plates were used as templates in standard PCR reactions with SP6 and T7 primers to amplify plasmid inserts. The sizes of the products were assessed on 1.5% agarose gels. Plasmids containing the insert were purified using plasmid isolation and purification Kit (Promega) and used as a template for DNA sequencing. Nucleotide sequence analysis was performed using the dideoxynucleotide chain termination method on a DNA sequencer (Model 373A, Applied Biosystems). Plasmid DNA at 1 µg was used for sequencing with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and subjected to electrophoresis on 6% denaturing gels. Clones were sequenced with the M13 forward and reverse primers. The SP gene sequence was analysed and compared by using the BLASTX and BLASTP search programs (<http://blast.genome.ad.jp>) with GenBank database search. Forward and reverse sequences were checked, and vector bases were trimmed using Chromas software (Version 2.13). Contiguous fragments were assembled using ContigExpress, part of the Vector NTI software package (Version 9). The multiple sequence alignment was created by using CLUSTAL W (Vector NTI package), and the same software was used to analyse similarity of the aligned sequences using a neighbour joining algorithm.

2.6. Expression of SP gene in tissues

Heart, gill, muscle, haemocyte, hepatopancreas, antennules, eyestalk, ovary and intestine were dissected out from mature *S. serrata*. The tissues were blotted and weighed then homogenised in Trizol reagent using a glass homogeniser. Total RNA was extracted as described above. The extracted and purified total RNA was quantitatively determined to the same concentration, and a sample of total RNA at 5 µg was used for reverse transcription. The first-strand cDNA was synthesised using a cDNA first-strand synthesis Kit with Superscript III reverse transcriptase (Invitrogen). Two gene-specific primers SPG 1F and SPG 1R were used to amplify the SP transcript and the primers β-actin F and β-actin R were used to amplify β-actin fragment that was used as a positive control (Table 1). The PCR reaction was the same as that described above.

2.7. SP expression of *S. serrata* under high pH stress, and injection with zymosan

The experiment was divided into two treatments representing different pH values (pH 8 and pH 9.5) in triplicate (three crabs in each replicate). *S. serrata* was incubated individually for 1, 3, 5 days in 10 litres of seawater (35‰) at pH 8 or 9.5. The pH of test media was adjusted with 0.5 N HCl and 0.5 N NaOH solutions. For the challenge test, there were two treatments (*S. serrata* injected with zymosan and crab injected with saline) combined with four exposure times (0, 6, 12 and 24 h). Zymosan, which had been dissolved in 0.85% NaCl solution to 5 mg ml⁻¹, was used as test solution. *S. serrata* were injected at the base of the right fifth leg with zymosan solution (around 200 µl) to reach a dose of 10 mg kg⁻¹. Crabs were injected with 200 µl saline as control. For each treatment and each exposure time, three crabs were sampled from the haemolymph. Seawater was replaced daily. Haemolymph of *S. serrata* in different pH and zymosan challenge was taken by inserting a syringe into the base of right chelate leg. Five millilitres of haemolymph were collected into a 25-ml test tube containing 5 ml of anticoagulant [21]. The diluted haemolymph was centrifuged at 500 × g at 4 °C for 20 min. The resulting pellet was used for total RNA isolation, and used for the SP transcript analysis by PCR as described above. The RT-PCR was performed using gene-specific primers SPG 1F and SPG 1R with cDNA (as a template) that had been reverse transcribed. The primers β-actin F and β-actin R were used to amplify β-actin fragment that was used as a positive control for RT-PCR.

All PCR products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide (100 ng ml^{-1}). After gel running, the DNA was visualised using an UV lamp. The expected size DNA fragment was excised in a minimal volume of agarose using a clean razor blade, and the gel slice was transferred to a micro-centrifuge tube. Each DNA fragment was isolated from agarose gel by Wizard[®] SV Gel and PCR Clean-Up System (Promega). The isolated DNA fragment was dissolved in DEPC-water, and the optical density (O.D.) at 260 nm was measured using a spectrophotometer (Model U-2000, Hitachi, Tokyo, Japan). Effects of pH and zymosan injection on the SP expression (SPE) were expressed as follows:

$$\text{SPE}(\%) = \{(\text{O.D. value of SP})/(\text{O.D. value of } \beta\text{-actin})\} \times 100$$

2.8. Statistical analysis

A multiple comparison (Duncan) test was conducted to compare the significant differences between different pH and between zymosan injection group and control group using SAS software (SAS Institute Inc., Cary, NC, USA). A significance level of $P = 0.05$ was chosen.

3. Results

3.1. Sequence of SP cDNA

The gene-specific primer pair SPG 1F and SPG 1R were used to amplify 390 bp fragment. (Fig. 2) of partial SP from haemocytes of *S. serrata*, and the fragment showed significant similarity to SP of other decapod crustaceans in GenBank database. Using similar approaches, 542 bp and 334 bp cDNA fragments were amplified by 5'- and 3'-RACE, respectively. The full-length SP cDNA fragment of *S. serrata* was obtained by overlapping three cDNA fragments.

The complete cDNA sequence and the deduced amino acid sequence of SP of *S. serrata* are shown in Fig. 3. The full-length of SP cDNA of *S. serrata* consisted of 1266 bp, including the start codon ATG at positions 19–21, the

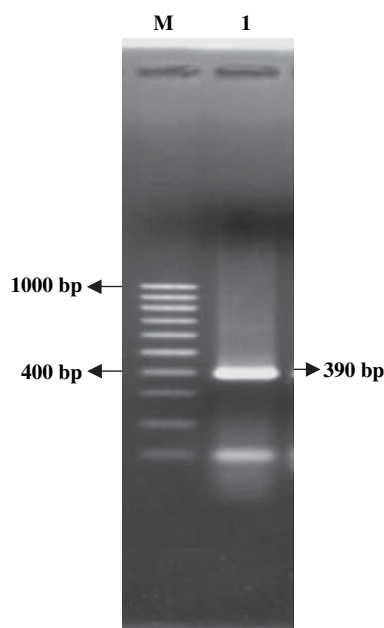


Fig. 2. PCR product amplified with *S. serrata* serine proteinase gene-specific primer SPG 1F and SPG 1R. Lane M, 100-bp ladder DNA marker, lane 1, cDNA (390 bp).

1	ACTGGAGCACGAGGACACATGTACTATCTTTCTTTCTCTCTGAACCTTGTTCCATTAATGTCCTAGAA	69
1	<u>M Y Y L F F L L N F V S I N V L E</u>	17
70	CTCCCGACCATAAACGAAACATGCACCGTCACCGGATGCGGTGAAACGGAACCAACGACCAAGCGAT	138
18	L P T I N E T C T V T G C G E T E T K R P S D	40
139	ATACAGTTGCACGTGGATTTCGATATCTGTATCTCTGCGTCTTGCAACCGATGTCGAGTTCACCGACGAA	207
47	I Q L H V D F D I C I L R L A T D V E F T D E	63
208	TTACGTCCCATATGCATACCGTTGGAGCTGGAAGTTCAGAAGAAGATCTGGGTGCCAGCCTGCTCTGCG	276
64	L R P I C I P L E L E V Q K K I W V P A C S A	86
278	CACCACCAGGCTCGGCAAGTTCTTTATCTGGACAAGTCTCTCTGCGCCGGCGGAGAGGCTGGGGA	345
87	H H Q A R Q V L Y P G Q V L P L R R R R G W G	109
346	CTCTGCGAGCGGCACGGAGATTCTCTCTGCGTTCCTGGACAAGACCAAGACCCAGTACGTGCCGCT	414
110	L L Q R R R R F S S G L P G Q D Q D P V R A G	132
415	TGGCATTTGGCGTGGGGCATTGGTTGCGGCACCTAGCACGACTGCCTCGTCACCGGCTGCGGCAAGGA	483
133	W H C G V G H W L R H L A R L P R H R L R Q G	155
484	CAGGTTTGGCAACAAGGGAATCTACCAAACTGTGTTAAAGACGTACCTGCAAGAGGCTGGTTGTGGGA	552
156	Q V W Q Q G N L P N C V K D V P A K R L V V G	178
553	GTTGCCGACCACAACATGCAGTCCACCAACGATGACGAACAGGAGTACCCGCTGGTGAGCGTTTCAG	621
179	V A D H N M Q S T N D D E P G V T R L V S V Q	201
622	GACATTACCGTTATCCTGACTATAATTCCTGACCTTGACAGTGATATCGCGCTAATTACGCTGTCA	690
202	D I T V H P D Y N S R T L D S D I A L I T L S	224
691	GAAACACTGGACCTGACTCAACACAAGGAGCTTCGCCCTGTGTCTGCCTGCCGATGACTCCAAGACT	759
225	E T L D L T Q H K E L R P V C L P A D D S K T	247
760	TATGACAGGCATGATGGCCACAGCGACAGGCTGGGGCACACTCCAGTCCGGGGGAGAGCGACCATATATA	828
248	Y A G M M A T A T G W G T L Q S G G E R P D I	270
829	CTGAATGAAGTCTCAGTACCCATTCTGGAACCTCTGCCCGGAAATGGATATCACTGAAAAATATGCTG	897
271	L N E V S V P I L E P S C P E M D I T E N M L	293
898	TGTGCTGGTCTTGAGGAAGGTGGTAAGGACACCTGTGGTCTTGAGGAAGGTGGTAAGGACACCTGCCAA	966
294	C A G L E E G G K D T C G L E E G G K D T C Q	316
967	GGTGACTCCGCGGCTCCGCCTCATGACACCTGCCAGGGAGACTCCGGCGGCCCGCTGTACGTGGAGGAA	1035
317	G D S G G P P H D T C Q G D S G G P L Y V E E	339
1036	AACTCCGTGCGAGTGCAGGTCCGCATCACCTCGTGGGGCTACGGGTGTGCTGACGCAACAGTCCCGGG	1104
340	N S V R V Q V G I T S W G Y G C A D A N S P G	362
1105	GTGTACGCCAGGGTCAGCAAGTACGTGTCCTGGATCAAGGAATAACACTCAGGACGCCACCACCTGCCA	1173
363	V Y A R V S K Y V S W I K E #	376
1174	GTAGAGCGTCGCTCACACTCCCTCTGACAACACGCACTGCATACCTTTCTTGGCGCAATAAAGCGGC	1242
1243	ACCAACCTGAAAAAAAAAAAAAAAAA	1266

Fig. 3. Nucleotide sequence (above) and deduced amino acid sequence of the open reading frame (below) of *S. serrata* putative serine proteinase (SP) cDNA. Nucleotides are numbered from the first base at the 5' end. Amino acids are numbered (italics) from the initiating methionine. The conserved SP domain is boxed. Conservative catalytic triad H, D, S is bold and boxed. The signal peptide is denoted by a double underline. The polyadenylation signal AATAAA is single-underlined. Arrowheads indicate the forward and reverse primers SPG 1F and SPG 1R, respectively. The sequence was submitted to GenBank with accession number AY946200.

termination codon TAA at positions 1147–1169, a potential polyadenylation signal, AATAAA at positions 1231–1236 and a poly A tail. The open reading frame (ORF) was 1131 bp beginning at nucleotide 19 bp and ending at nucleotide 1149 bp. Based on the deduced polypeptide sequence, the open reading frame of SP cDNA was found to be composed of 376 amino acids, including a 16 amino acids signal peptide sequence predicted by SignalP software (ExPASy) in the N-terminal region of the polypeptide chain. Therefore, the catalytic domain of mature SP is composed of 360 amino acids residues. Amino acids are numbered beginning with the first methionine (Fig. 3). The calculated molecular mass of serine proteinase mature protein is 39.54 kDa with an estimated *pI* of 5.37. The

cDNA sequence and deduced amino acid sequence has been submitted to the NCBI GenBank as accession number [AY946200](#).

3.2. SP domain of *S. serrata*

The putative deduced amino acid sequence showed one C-terminal trypsin like SP domain from residue 166 through 376 (Fig. 3). The deduced 210-amino-acid sequence showed high homology to a conserved domain (NCBI: cd00190) for a trypsin-like serine proteinase, a universal C-terminal feature of PPAF-serine proteinases. Downstream from the stop codon was a 117 bp 3'-UTR containing a typical polyadenylation signal (AATAAA). The typical catalytic triad of serine proteases required for functional activity (His150, Asp217 and Ser331) was conserved in the polypeptide sequence of *S. serrata*. The sequence around the catalytic residues also exhibits the highest overall sequence identity in the SP domain. The sequence showed five cysteine residues (Cys125, Cys239, Cys283, Cys327 and Cys355) at suitable positions, and these Cys are forming two disulphide bridges: Cys239–Cys283 and Cys327–Cys355. One Cys residue adjacent to His150 is missing. Comparison of cDNA and amino acid sequences of SP as deduced from GenBank by BLAST and SMART (EMBL) revealed that a trypsin-like serine proteinase domain showed a high sequence similarity with other SP (Fig. 4). The most similar sequences were from spiny lobster *P. argus* ([AF357226](#)), fruit fly *D. melanogaster* ([L11451](#)), crayfish *P. leniusculus* ([AJ007668](#)), centipede *Scolopendra subspinipes* ([U79521](#)), honey bee *Apis mellifera* ([XP_392559](#)), and African malaria mosquito *Anopheles gambiae* ([Z49813](#)). A search in the GenBank database, using the BLASTP program, revealed that the deduced sequence *S. serrata* SP was similar to SP from other arthropods (Table 2).

3.3. Expression analysis of *S. serrata* SP mRNA

The primer set used for quantitative PCR (SPG 1F and SPG 1R) was designed to the putative SP sequence to yield a 390 bp product within the conserved domain (Fig. 5A). All reactions using β -actin as the positive control were detected in all tissues (Fig. 5B). A predominant SP transcript expression with the expected size was observed only in haemocytes and weak expression was detected in heart, eyestalk and antennules, but not in other tissues (Fig. 5A).

3.4. SP expression of *S. serrata* under high pH stress and injected with zymosan

The primer combination was examined in vivo in *S. serrata* SP transcription. All PCR products of *S. serrata* exposed to different pH and all PCR products of *S. serrata* challenged with zymosan were 390 bp. Experiments results showed that SP expression slightly reduced after 1 day, and significantly decreased when *S. serrata* was exposed to pH 9.5 after 3 and 5 days (Fig. 6A). The SP transcript significantly increased after 24 h for the *S. serrata* injected with zymosan at a dose of 10 mg kg⁻¹ (Fig. 6B).

4. Discussion

The full-length sequence of 1266 bp SP cDNA was cloned from the haemocytes of *S. serrata*. The open reading frame encodes a protein of 376 amino acids with a signal sequence of 16 amino acids. GenBank homology search and CLUSTAL W multiple sequence alignment confirmed that the cloned cDNA sequence of *S. serrata* encodes a SP domain. This sequence contains the conserved residues of the putative active site, His150, Asp217 and Ser331, which form the characteristic SP catalytic triad found in crustaceans [16,17,27]. Furthermore, the sequence alignment of *S. serrata* SP revealed that the predicted amino acid sequence was homologous to other arthropod trypsin-like SP in the NCBI GenBank and showed similarity of 55%, 51% and 50% to SP from spiny lobster *P. argus*, horseshoe crab *T. tridentatus* and crayfish *P. leniusculus*, respectively (Fig. 4). The facts that the homology of *S. serrata* SP sequence with other amino acid sequence and that the expression pattern are consistent suggest that *S. serrata* SP is involved in the activation of prophenoloxidase.

Serine proteinases, the most studied class of proteinases, belong to a diverse multigene family that share a common catalytic mechanism and structural characteristics such as the presence of three conserved amino acid residues, His, Asp and Ser in the active site. These enzymes are involved in several biological processes in higher animals, including digestion, proenzyme, prohormone, and complement activation, as well as participating in defence mechanisms [4,5].

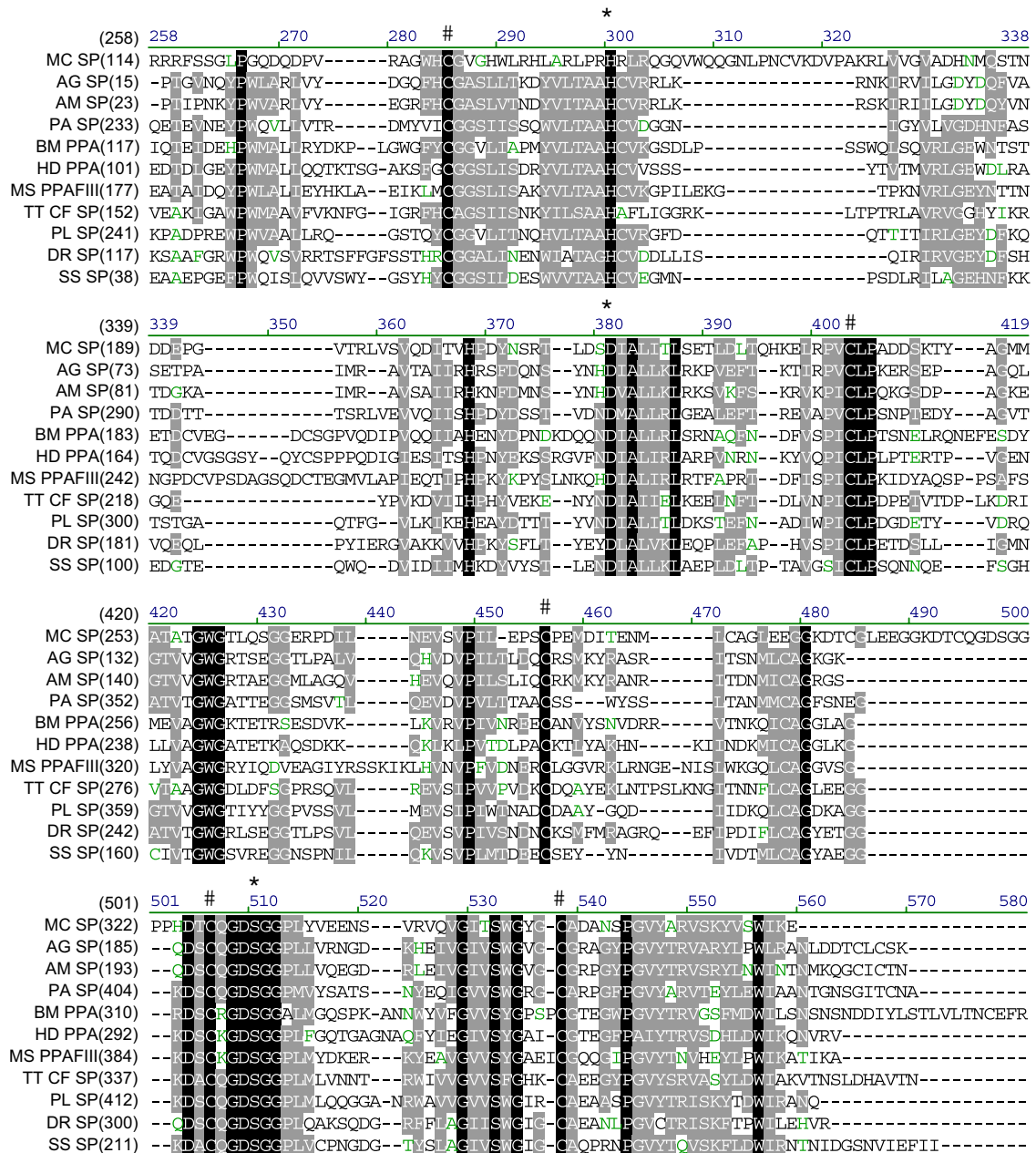


Fig. 4. Multiple alignment of amino acid sequences of serine proteinase: sequences were from MC SP (mud crab *S. serrata* SP; AY946200), AG SP (*Anopheles gambiae* SP; Z49813), AM SP (*Apis mellifera* SP; XP_392559), PA SP (*Panulirus argus* SP; AF357226), BM PPA (*Bombyx mori* Masquerade-like serine proteinase homologue; AF513368), HD PPA (*Holotrichia diomphalia* prophenoloxidase activating factor-III; AB079666), MS PPAFIII (*Manduca sexta* serine protease; AAV91015), TT CF SP (*Tachyplesus tridentatus* Coagulation factor serine protease), PL SP (*Pacifastacus leniusculus*; AJ007668), DR SP (*Drosophila melanogaster* transmembrane serine proteinase; L11451) and SS SP (*Scolopendra subspini* plasminogen activator serine protease; U79521). Black indicates complete conservation. Grey shading indicates conservation replacements. # indicates conserved cysteine residues. * indicates conserved catalytic triad.

The domain structure of SP of *S. serrata* is similar to that of the serine proteinase of coleopteran *H. diomphalia*, spiny lobster *P. argus* and horseshoe crab *T. tridentatus*. These are the members of the clip domain SP family with a carboxyl-terminal SP domain and an amino-terminal clip domain. Clip domains were first identified in the proclotting enzyme of horseshoe crab *T. tridentatus* and have also been found in many arthropod SPs that play a role in embryonic

Table 2
Similarity comparison of mud crab *Scylla serrata* serine proteinase with other species

Species	Serine proteinase protein	GenBank accession no.	% identity/similarity
Spiny lobster <i>Panulirus argus</i>	Trypsin like serine protease	AF357226	42/55
Centipede <i>Scolopendra subspinipes</i>	Plasminogen activator serine protease	U79521	37/54
Horseshoe crab <i>Tachypleus tridentatus</i>	Coagulation factor serine protease	D14701	37/51
Fruit fly <i>Drosophila melanogaster</i>	Transmembrane serine proteinase	L11451	37/51
Crayfish <i>Pacifastacus leniusculus</i>	Serine protease	AJ007668	34/50
African malaria mosquito <i>Anopheles gambiae</i>	Serine proteinase	Z49813	35/50
Honey bee <i>Apis mellifera</i>	Serine proteinase	XP_392559	34/50
Coleopteran <i>Holotrichia diomphalia</i>	Prophenoloxidase activating factor-III serine proteinase	AB079666	31/50
Domestic silkworm <i>Bombyx mori</i>	Masquerade-like serine proteinase homologue	AF513368	31/47
Tobacco hornworm <i>Manduca sexta</i>	Serine protease	AAV91015	30/43
White shrimp <i>Litopenaeus vannamei</i>	Trypsin-like serine proteinase	AY368151	28/44
Kuruma shrimp <i>Marsupenaeus japonicus</i>	Serine proteinase homologue	AB161692	27/43
Tiger shrimp <i>Penaeus monodon</i>	Trypsin-like serine proteinase	AY372186	26/42
Mud crab <i>Scylla serrata</i>	Serine proteinase	AY946200	Present study

development and immune responses [5]. Clip domains regulate catalytic domain activity by interacting with substrates or regulatory molecules [28,29]. In the present study, by comparing the sequence with canonical clip domains, the SP sequence showed five cysteine residues (Cys125, Cys239, Cys283, Cys327 and Cys355) at comparable positions. In general, SP has six conserved cysteine residues forming disulphide bridges [5,28,30], only five were found in *S. serrata* SP and it is likely that these Cys are forming two disulphide bridges: Cys239–Cys283 and Cys327–Cys355 at positions consistent with the clip domain as it was the first described [29,30]. The clip domain of *S. serrata* SP showed low sequence similarity with that of other members of the clip domain SP family and was found to have only five Cys

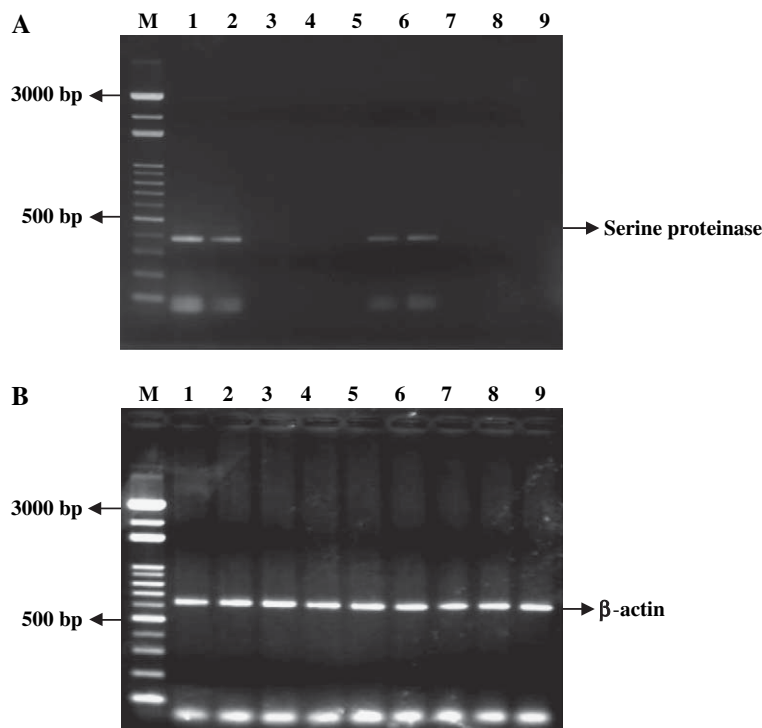


Fig. 5. Expression of mud crab *S. serrata* SP in various tissues (A). β -Actin was used as control in all tissues (B). M indicates the molecular weight marker. Lane 1, haemocytes; lane 2, heart; lane 3, gill; lane 4, muscle; lane 5, hepatopancreas; lane 6, antennules; lane 7, eyestalk; lane 8, ovary; lane 9, intestine.

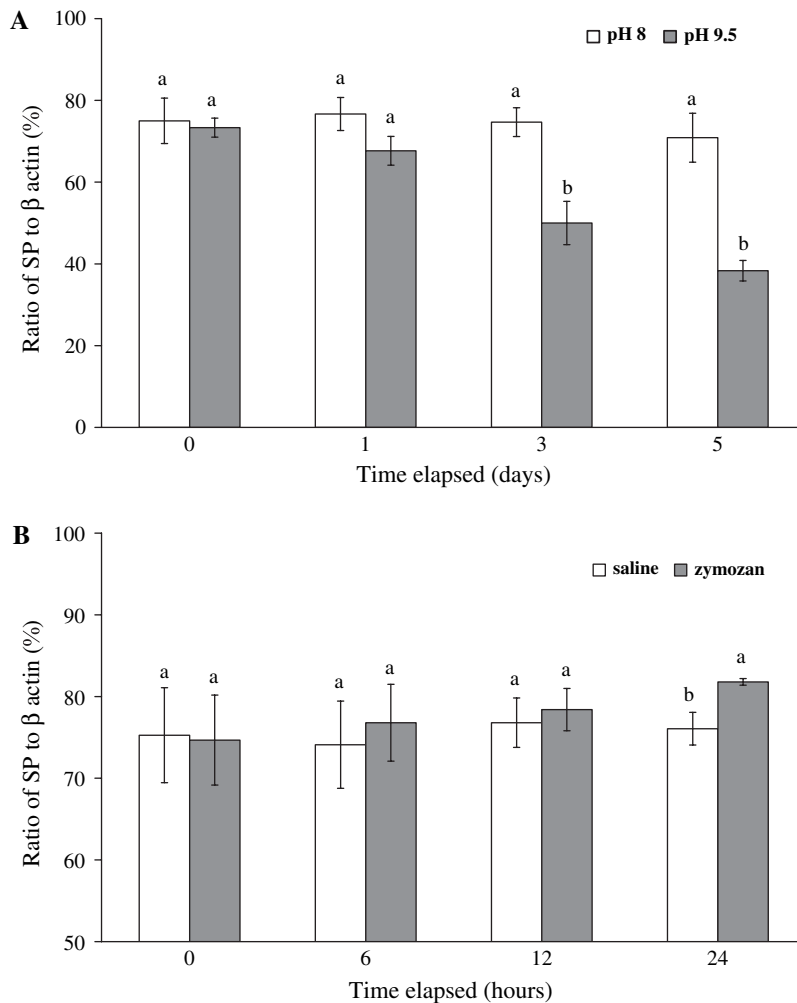


Fig. 6. Serine proteinase in the haemocytes of *S. serrata* subjected to different water pH (A), and SP in the haemocytes of *S. serrata* injected with saline (control) and zymosan (B). Each bar represents mean value with standard error ($n = 3$). Bar in the same exposure time with different letters are significantly different ($P < 0.05$) between treatments.

residues. Hence, it is only a partial clip domain, similar to those found in SPs of fruit fly *D. melanogaster* [10], domestic silkworm *B. mori* [24], spiny lobster *P. argus* [19] and kuruma shrimp *M. japonicus* SPs [16].

Sequence comparison of SP of *S. serrata* with SP of tiger shrimp *P. monodon* (AY372186), white shrimp *L. vannamei* (AY368151) and kuruma shrimp *M. japonicus* (AB161692) showed low similarity (below 45%). Although these three species are decapods, the sequence identities among them are not as high as compared to other biodefence genes such as peroxinectin [31] and lipopolysaccharide- and β -1,3-glucan binding protein (LGBP) [32] with similarities of approximately 90%. This fact indicates that SP genes are structurally diverse in decapod crustaceans and such diversity has implications for the variety of their biological functions [4]. Further study is needed to understand the relationship of this diversity with corresponding biological functions.

In the present study, SP transcript is expressed predominantly in haemocytes and encodes a protein involved in immune responses, however with weak expression in heart, antennules and eyestalk. Serine proteinase gene is abundant in the antennules lateral flagella and eyestalk of spiny lobster *P. argus* [19]. In insect, tobacco hornworm *M. sexta* PPAF-I, PPAF-II, and PPAF-III and several immune-related SPs of mosquito *A. gambiae* were expressed in haemocytes and/or fat body, which are two major expression sites of immune-related proteins [28,33–35]. Masquerade-like SPH and PPAF mRNA are also expressed in haemocytes of crayfish *P. leniusculus* [36]. These facts suggest that SP produced in haemocytes is the major site for immune-related SPs synthesis. Furthermore, haemocytes are systemically

circulated, thus they are able to target pathogenic agents, release biodefence genes or activate the host's biodefence mechanisms.

In decapod crustacean, it is known that environmental changes may affect the immune ability leading to susceptibility against pathogen infection. The present study indicated that SP expression was higher in haemocytes of *S. serrata*, and was significantly reduced for the crab exposed to pH 9.5 after 3 and 5 days, indicating suppression of immunity. Giant freshwater prawn *Macrobrachium rosenbergii* exposed to high pH decreased its phenoloxidase activity indicating a reduction in immune ability [37]. In the present study, SP transcript increased 24 h post-zymosan injection. The serine proteinase homologue expression of kuruma shrimp *M. japonicus* dramatically increased after 3 days feeding with peptidoglycan at 0.2 mg kg⁻¹ shrimp body weight per day [16]. Moreover, the synthesis of SP from *L. vannamei* was not stimulated by inoculation of *Vibrio alginolyticus* [17]. This discrepancy is considered due to the number of SP gene expression and the number of newly synthesised haemocytes.

In conclusion, a 1266 bp serine proteinase cDNA cloned from the haemocytes of *S. serrata* indicates that SP is synthesised in haemocytes. It encodes 376 amino acids (a mature protein of 360 amino acids). The calculated molecular mass of the SP mature proteins is 39.54 kDa with an estimated pI of 5.37. The SP deduced amino acid sequence of *S. serrata* has similarity of 55%, 51% and 50% to SP amino acid sequence from spiny lobster *P. argus*, horseshoe crab *T. tridentatus* and crayfish *P. leniusculus*, respectively. The SP transcript of *S. serrata* following 3 days exposure to pH 9.5 decreased significantly as compared to that of crab at pH 8. However, the SP transcript increased significantly 24 h post-zymosan injection.

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