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Isolation, gene cloning and expression profile of a pathogen recognition protein: A serine proteinase homolog (*Sp*-SPH) involved in the antibacterial response in the crab *Scylla paramamosain*

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ABSTRACT

To identify the frontline defense molecules against microbial infection in the crab *Scylla paramamosain*, a live crab pathogenic microbe, *Vibrio parahaemolyticus*, was recruited as an affinity matrix to isolate innate immune factors from crab hemocytes lysate. Interestingly, a serine proteinase homolog (*Sp*-SPH) was obtained together with an antimicrobial peptide-antilipopolysaccharide factor (*Sp*-ALF). We then determined the full-length cDNA sequence of *Sp*-SPH, which contained 1298 bp with an open reading frame of 1107 bp encoding 369 amino acid residues. Multiple alignment analysis showed that the deduced amino acid sequences of *Sp*-SPH shared overall identity (83.8%) with those of SPH-containing proteins from other crab species. Tissue distribution analysis indicated that the *Sp*-SPH transcripts were present in various tissues including eye stalk, subcuticular epidermis, gill, hemocyte, stomach, thorax ganglion, brain and muscle of *S. paramamosain*. The *Sp*-SPH was highly expressed in selected different development stages including embryo (I, II, III and V), zoea (I), megalopa, and juvenile. Importantly, the prophenoloxidase was also present in the embryos, zoea, juvenile and adult crabs, but relatively lower in megalopa compared to those of other stages. Furthermore, the *Sp*-SPH mRNA expression showed a statistically significant increase ($P < 0.05$) in both hemocyte and subcuticular epidermis at 24 h, and in gill at 96 h after challenge of *V. parahaemolyticus* determined by quantitative real-time PCR. Taken together, the live-bacterial-binding activity and the acute-phase response against bacterial infection of *Sp*-SPH suggested that it might function as an innate immune recognition molecule and play a key role in host defense against microbe invasion in the crab *S. paramamosain*.

1. Introduction

The crab *Scylla paramamosain* is an important cultured crustacean in China and Pacific areas which contributes a lot to the local economy. Whereas, the disease epidemics have resulted in a dramatic mortality due to high densities of crab culture and have caused a severe economic loss. Previous study has shown that *S. paramamosain* mounts an strong immune response against LPS challenge by using both humoral and cellular responses as shown by a global screening of immune genes from its hemocytes [1]. However, the knowledge about pathogen recognition and innate immune response against pathogenic bacteria in cultured crabs is still limited. Invertebrates including crab rely solely on multiple innate immune defenses to combat microbial infections. These immune responses are triggered via recognition of pathogen molecules (like LPS/PGN/LTA from bacterial components, β-1,3-glucan from fungal cell walls and dsRNA of viruses) not present in the host by pattern recognition proteins (PRPs) [2]. Current studies have identified many PRRs such as LPS or/and β-1,3-glucan binding proteins (LBP, βGBP or LGBP), peptidoglycan recognition proteins (PGRPs), and lectins from various invertebrates [3]. After binding to microbes, these PRRs will initiate different biological processes like activation of prophenoloxidase (proPO)-system and release of antimicrobial peptides (AMPs).

The melanization reaction initiated by activation of proPO-system plays key roles in host defense against microbial infections in both insects and crustaceans [4–8]. Recent studies have demonstrated that clip domain serine proteinases act as important cofactors for the activation of proPO cascade in arthropods [9,10]. Besides, the non-catalytic clip domain serine proteinase homologues (SPHs), which do not possess protein hydrolytic activity by non-synonymous substitution of one or several critical amino acids in the catalytic triad, have also been studied as key molecules involved in the activation/regulation of the proPO-system in insects such as *Anopheles* [11], *Holotrichia diomphalia* [12–15], *Tenebrio*

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molitor [16,17], *Manduca sexta* [18–22], and *Cotesia rubecula* [23]. In crustaceans, a number of SPH-containing proteins were characterized with different biological functions like cell adhesion activity [24–26], binding to virus [27], action in antimicrobial infection [28], and functioning in defense triggered by immune challenges [29–31]. Recently, two SPHs were also isolated from different crab species and they were proposed to function as important factors involved in tanning or sclerotization in *Callinectes sapidus* [32], and in acute-phase defense against bacterial challenge in *Eriocheir sinensis* [33]. However, no functional study on SPHs was carried out from the two crab species mentioned above and another isolated SPH (ACN87221) from *Portunus trituberculatus*. Hence, it is necessary to elucidate any biological function concerning the SPH-containing proteins for disease control in the farmed crabs.

As we know, hemocytes play critical roles in the frontline defense in crustaceans like crab, and how the hemocytes are triggered to release the defense factors in vivo in response to various pathogen intruders, especially the pathogenic bacteria, remains largely unknown in the crab *S. paramamosain*. Generally, hemocytes contain a large number of immune factors which are critical for pathogen sensing and immune signal transduction. Meanwhile, *Vibrios* is well-known for its highly pathogenicity against many aquatic species, which has caused a huge economic loss in farmed crabs [34]. To characterize the pathogen recognition proteins from hemocyte lysate supernatant (HLS) of *S. paramamosain*, a live-bacterial-affinity matrix was recruited to isolate effector proteins directly interacting with a crab pathogenic bacterium *Vibrio parahaemolyticus* in this study. A SPH protein (*Sp*-SPH) was then isolated from crab HLS by its binding activity to *V. parahaemolyticus*. Furthermore, full-length cDNA cloning, mRNA expression distribution in adult crab, mRNA expression (both *Sp*-SPH and *Sp*-proPO) in different development stages, and mRNA expression profile of *Sp*-SPH in hemocyte, subcuticular epidermis and gill post-challenge of *V. parahaemolyticus* were also determined.

2. Materials and methods

2.1. Crab HLS and plasma preparation

Live healthy inter-molting male crabs, *S. paramamosain* (200 ± 50 g), were bought from a local commercial crab farm in Xiamen, China. The haemolymph samples were prepared as previously described [1]. Briefly, the haemolymph was collected and mixed with crab anticoagulant solution (NaCl 510 mM; glucose 100 mM; citric acid 200 mM; Na-citrate 30 mM; EDTA-Na_2 10 mM; pH 7.3) [35] (1:1) on the ice followed by centrifugation at 800 × *g* for 10 min at 4 °C. The supernatant was employed as crab plasma solution. The resulting hemocyte pellets were then suspended in crab anticoagulant solution (2 ml/crab) and sonicated on the ice. After sonication, the mixture was subjected to centrifugation at 15,000 × *g* for 10 min at 4 °C. The supernatant was then collected and used as HLS for further experiments. The protein concentrations were determined by Bradford method as previously described [36].

2.2. Microbial organism preparation

V. parahaemolyticus, a marine pathogenic bacterium for *S. paramamosain*, was cultured in 2216E medium overnight at 28 °C. For binding experiments, fresh culture was prepared by inoculation of an overnight culture (1:100) into fresh 2216E medium for further culturing till the density of 0.5–0.6 at $OD_{600\,nm}$.

2.3. Extraction of proteins from crab HLS and plasma using live microbes as affinity matrix

For isolation of proteins recognizing *V. parahaemolyticus*, the experiments were performed according to a previous study [37]. Briefly, the fresh cultured "bacterial-beads" above were washed repeatedly with crab saline (NaCl, 496 mM; KCl, 9.52 mM; $MgSO_4$, 12.8 mM; $CaCl_2$, 16.2 mM; $MgCl_2$, 0.84 mM; $NaHCO_3$, 5.95 mM; HEPES, 20 mM; pH 7.4) [38] followed by centrifugation at 1,800 × *g* for 3 min at 4 °C. The washed "bacterial-beads" were thoroughly resuspended in 1/20 of original volume of crab saline and added to HLS or plasma at the density of the original culture. After incubation with rotation at 25 °C for 20 min, the "bacterial-beads" were pelleted by centrifugation and washed twice with crab saline. The bacterial pellets were then resuspended in 1/5 of original volume of extraction buffer instead of crab saline under the following conditions for 15 min with mild rotation: (1) 4 M urea, in 10 mM Tris–HCl, pH 8.0; (2) 0.1 M citric acid, pH 2.0; (3) 0.15 M triethylamine, pH 11.5. The "bacterial-beads" incubated with crab saline and crab HLS alone or crab plasma alone were also prepared as control treatments and subjected to the same elution treatments as described above. The extracted proteins in the supernatant were then separated from the pellets by centrifugation at 15,000 × *g* for 2 min at 4 °C. The supernatants containing the eluted binding proteins were transferred into new tubes and concentrated by adding 1/10 volume of trichloroacetic acid and kept on ice for 30 min followed by centrifugation at 15,000 × *g* for 3 min. The resulting protein pellets were resuspended directly in 1× SDS-PAGE loading buffer and 20 μl of each eluted samples was prepared for 15% SDS-PAGE under denaturing conditions.

2.4. Determination of partial peptide sequences of bacterial-binding proteins

The partial internal peptide sequences of bands A–E proteins were subjected to mass spectrum (LTQ-MALDI-TOF/TOF-MS, APT Co., Shanghai, China) analysis. The peptide fragments obtained were shown in Table 1. Two degenerate

Table 1
Identification of *V. parahaemolyticus* binding proteins by LTQ-MOLDI-TOF/TOF-MS.

Bands no.	MS-BLAST HIT	Fragment sequences	Fragment numbers
A	PAF, *P. trituberculatus*	K.GVFQNVLK.K	1
		R.VRLG***EWDTQNEY***EPYK.H	2
	PAF, *C. sapidus*	K.SFLCA***GGEAGK.D***	4
B	No reliable hit	ND	0
C	PAF, *C. sapidus*	K.SFLCA***GGEAGK.D***	3
	PAF, *P. trituberculatus*	K.GVFQNVLK.K	2
D	PAF, *C. sapidus*	K.SFLCA***GGEAGK.D***	4
	PAF, *P. trituberculatus*	K.GVFQNVLK.K	1
E	ALF, *P. trituberculatus*	K.FWCPGWAPFEGR.S	3
		K.LITENNAAAWLKG	2

PAF, phenoxidase activating factor; ALF, antilipopolysaccharide factor; ND, not determined. Peptide fragments for designing degenerate primers were in bold and italic.

primer pairs (5′-GARTGGGAYACNCARAAYGARGT-3′ and 5′-ACYTCRTTYTGNGTRTCCCAYTC-3′ based on EWDTQNEY for 3′ and 5′ RACE, respectively; 5′-GGNGGNGARGCNGGNAARGA-3′ and 5′-TCYTTNCCNGCYTCNCCNCC-3′ based on GGEAGK.D for 3′ and 5′ RACE, respectively) were designed for RACE-PCR amplification to determine full-length cDNA sequcence of *Sp*-SPH.

2.5. Cloning of the full-length cDNA of Sp-SPH gene

To obtain the full-length cDNA of *Sp*-SPH gene, 3′-RACE and 5′-RACE were carried out. Total RNA was extracted from samples (healthy crab hemocytes) using Trizol reagent following the manufacturer's instructions and quantified with an Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Sweden). And the cDNA was synthesized by Super SMART™ PCR cDNA Synthesis Kit (Clontech). The degenerate primers for *Sp*-SPH gene cDNA were designed according to the obtained partial peptide sequence as shown in Table 1. The RACE cDNA was prepared with an SMART RACE cDNA Amplification kit (Clontech, USA) according to the manufacturer's protocol. And the PCR conditions were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 40 s, 62 or 58 °C for 45 s and 72 °C for 40 s; followed by the final extension at 72 °C for 7 min. The expected DNA fragment was extracted from agarose gel and ligated to pMD18-T vector (Takara). The ligation product was transformed into *E. coli*. Then the recombinant clones were screened by bacterial colony PCR as previously described [1]. The selected positive recombinant clones were then sequenced at Shanghai Genewindows Biotech Co. Ltd (China). The resulting sequences were verified and subjected to cluster analysis.

2.6. The transcripts distribution of Sp-SPH in various tissues and different development stages of the crab

Haemolymph of three healthy male crabs (about 200 ± 50 g in weight per crab) was taken separately. Two millilitres of haemolymph per crab was individually collected into an equal volume of anticoagulant solution followed by centrifugation at 800 × *g* at 4 °C for 20 min. The resulting hemocyte pellet was used for total RNA isolation. Other tissues including brain, eye stalk, gills, heart, hepatopancreas, mid-gut gland, muscle, reproductive tract, stomach, subcuticular epidermis, and thoracic ganglion mass were also dissected and prepared for total RNA isolation as described above. Different development stages of the crab including whole embryo (I, II, III and V), zoea (I), and megalopa were used for total RNA isolation. Hemolymph from juvenile (10 ± 2 g, and 35 ± 5 g) and adult (200 ± 50 g) crab were also prepared as mentioned above. The cDNA synthesis was performed as previously described [1]. Quantitative real-time PCR was performed in a reaction mixture of 20 μl containing 0.5 ng of total transcribed cDNA, 5 pmol of each gene-specific primer and 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, UK). The standard cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Data of raw relative quantification were calculated using 7500 system SDS software version 1.3.1.21 and the actin gene was employed as the internal standard as previously described [1]. ANOVA comparison tests were employed for statistical analysis by SPSS software (version 11.5). Values were considered to be significant as $P < 0.05$.

2.7. The transcripts distribution of Sp-proPO in different development stages of the crab

To determine if *Sp*-proPO gene transcript was present in different development stages of the crab, the proPO primers (5′-GCTGCTGCCATTTGATGTCTC-3′ and 5′-CGAGAAGGTCAATTCCACGC-3′) were designed according to the proPO mRNA sequence reported from *Scylla serrata* (Genbank accession no. DQ435606). The cDNA from different development stages were prepared as above and used for semi-quantitative RT-PCR. The crab actin gene was employed as internal control. The PCR cycling program was: 94 °C for 3 min, followed by 33 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 20 s with a final extension at 72 °C for 7 min. The PCR products were then analyzed by 2% agarose gel stained with ethidium bromide.

2.8. The mRNA expression profile of Sp-SPH against challenge of V. parahaemolyticus in hemocyte, subcuticular epidermis and gill of the crab

V. parahaemolyticus was prepared and washed as described above for animal challenge. For the gene expression post bacterial challenge, 18 healthy male crabs were injected with a dose of 6×10^4 CFU mixed with 100 μl crab saline at the base of the right fourth leg, and the other 18 individuals were injected with an equal volume of sterile saline solution as control treatments. The crabs for each group (three crabs per group) were separately reared in individual tank under the same culture conditions. Sampling was performed at different time intervals (3, 6, 12, 24, 48 and 96 h) after bacterial challenge. Haemolymph was collected as described above and hemocyte pellets were preserved in Trizol reagent (Invitrogen) immediately for RNA extraction. Samples from the gill, subcuticular epidermis and hepatopancreas were also separately collected from each individual animal and were separately frozen immediately in liquid nitrogen, and stored at −80 °C for later use. The total RNA isolation, real-time PCR quantification and data analysis were performed as described above.

3. Results

3.1. Isolation of pathogen recognition proteins from crab HLS

To isolate the frontline recognition and defense factors from the crab *S. paramamosain*, a live crab pathogenic bacterium, *V. parahaemolyticus*, was used as "bacterial-beads" to fish bacterial-binding proteins from the HLS of *S. paramamosain*. Bound HLS proteins associated with the bacteria were eluted with different buffers and then analyzed by 15% SDS-PAGE under reducing conditions. For control treatment, *V. parahaemolyticus* was incubated with crab saline instead of the crab HLS. Crab HLS alone were also prepared as a control to avoid the false-positive protein

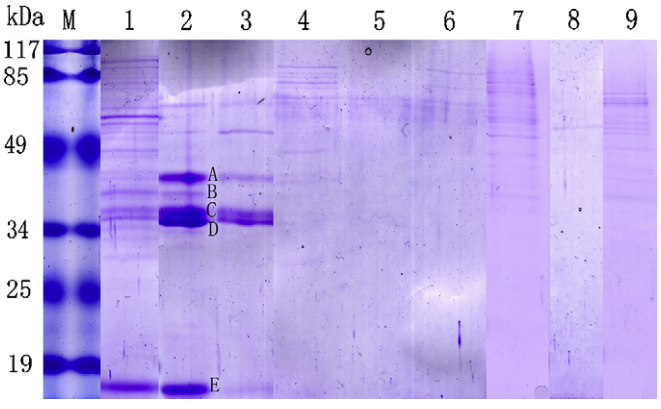

Fig. 1. Isolation and identification of pathogen recognition proteins from HLS of crab *S. paramamosain*. Live *V. parahaemolyticus* binding proteins were recovered by elution from bacterial cells incubated with the crab HLS under different elution treatments. (1–3) Bacteria + HLS; (4–6) HLS alone; (7–9) bacteria alone. (1, 4 and 7) Eluted with 4 M urea pH 8.0; (2, 5 and 8) eluted with 0.1 M citric acid pH 2.0; (3, 6 and 9) eluted with 0.15 M triethylamine pH 11.5. This experiment was repeated for three times.

precipitation during the experiments. As shown in Fig. 1, five bands with molecular masses ranging from about 34 to 45 kDa (A–D) and a smaller size of about 14 kDa (E) were present in SDS-PAGE gel in all three different elution solvent. Better elute was observed in 0.1 M citric acid (pH 2.0) treated samples compared to the other two solvents (urea and triethylamine). When eluted by citric acid, no obvious protein could be eluted from *V. parahaemolyticus* itself or crab HLS compared to the bands present in lane 2. This result clearly indicated that the *V. parahaemolyticus* binding proteins were from crab HLS and not from the bacteria or precipitation of crab HLS. In contrast, no obvious band was observed from the crab plasma preparation (data not shown). The observed major protein bands of interest from citric acid extracts were then prepared in-gel-digestion with trypsin and further analyzed by MS.

3.2. Determination of partial peptide sequences of bacterial-binding proteins

The proteins identified by LTQ-MALDI-TOF/TOF-MS were summarized in Table 1. Protein bands A, C and D were characterized to be the homolog of prophenoloxidase activating factor (PPAF) from crab *P. trituberculatus* (ACN87221), *C. sapidus* (AAS60227) and *E. sinensis* (ACU65942), which is a PPA factor without catalytic enzyme activity usually named as a SPH homolog. Hence, the A, C, and D were defined as a *Sp*-SPH in this study. Band E exhibited matching to antilipopolysaccharide factors (ALF) from *P. trituberculatus* (ACM89169), *S. paramamosain* [39], and *S. serrata* [40]. However, no signal was acquired for band B, which was not succeeded by the MS determination.

3.3. Cloning of the full-length cDNA sequence of Sp-SPH gene

To further determine the full-length cDNA sequence of *Sp*-SPH (the description of *Sp*-ALF is under preparation for another manuscript and would not be discussed in this study), degenerate primers based on the partial peptide sequences of EDWTQNEY and GGEAGKD were designed for 5′ and 3′ RACE PCR as described above and they were employed to amplify full-length cDNA sequence of *Sp*-SPH from crab hemocytes. As shown in Fig. 2, the cDNA sequence

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1    GGCAGTTGGCTGGACGGCAAGCGAACAGAAGGTGCCGCAAACTGTTTCCATCAGATCAGC
61   TTTCTATCCGCTCTGAAGATGCGACACCTGGCAGTCCTTGCCGCCCTAGTGGCCCTGGCC
1                        M  R  H  L  A  V  L  A  A  L  V  A  L  A
121  GCCGCTGGACCAAGGGAGCGGCGCCAGACCACCGAGTACGAAAGATGCCAGGACGGTACC
15   A  A  G  P  R  E  R  R  Q  T  T  E  Y  E  R  C  Q  D  G  T
181  AAGGTGTGCGTGCCCTACTACCTGTGCCAGGACGGCAAAGTGATAACTGACGGCTCTGGA
35   K  V  C  V  P  Y  Y  L  C  Q  D  G  K  V  I  T  D  G  S  G
241  GTTATCGACATCAGAACTGCATCAGGGTGCAGTAACTACTTGGACGTCTGCTGCGCCGAT
55   V  I  D  I  R  T  A  S  G  C  S  N  Y  L  D  V  C  C  A  D
301  CCCCACACGACAGATCCCGTCACCCCCGACCCAGGCCACGTTTCTCGTTGCGGAGTTAGG
75   P  H  T  T  D  P  V  T  P  D  P  G  H  V  S  R  C  G  V  R
361  AATTACAATGGCATCGATGTCAGGATCCAAGGATTCCAGGGCAACGAGACCCAGGTGGCT
95   N  Y  N  G  I  D  V  R  I  Q  G  F  Q  G  N  E  T  Q  V  A
421  GAGTTCCCCTGGATGTCCGCTGTGCTCAAGAAGGAGGTAGTGTCAGGAGAGGAGATCAAT
115  E  F  P  W  M  S  A  V  L  K  K  E  V  V  S  G  E  E  I  N
481  CTGTACCTGTGCGGCGGTTCGCTCATCCACCCCTCCATCGTGCTGACGGCGGCTCATTGC
135  L  Y  L  C  G  G  S  L  I  H  P  S  I  V  L  T  A  A  H  C
541  GTCAACAAGCACCTATCCTCAGACCTTCGCGTCCGTCTGGGAGAGTGGGACACTCAGAAC
155  V  N  K  H  L  S  S  D  L  R  V  R  L  G  E  W  D  T  Q  N
601  GAGTATGAGCCATACAAGCACCAGGACCGCGACGTGAGTGCTGTTGTCATCCATCCCAGG
175  E  Y  E  P  Y  K  H  Q  D  R  D  V  S  A  V  V  I  H  P  R
661  TTCAACGGCAGCAACCTGCACAATGACTACGCGCTGCTGTACCTCCAGACGCCCGCCGAG
195  F  N  G  S  N  L  H  N  D  Y  A  L  L  Y  L  Q  T  P  A  E
721  CTCAGCAGGAACGTGGATGTCATCTGCCTGGACAACAACCCCACCATCCTCGCTCCCCAC
215  L  S  R  N  V  D  V  I  C  L  D  N  N  P  T  I  L  A  P  H
781  CACAACTGCCTCGTCACCGGCTGGGGCAAAGACAGGTTCGGCAAGAAGGGAGTCTTCCAA
235  H  N  C  L  V  T  G  W  G  K  D  R  F  G  K  K  G  V  F  Q
841  AATGTGCTGAAGAAGATCGACCTCCCTTACGTTCCACATGGGGAATGCCAGACTGCTCTG
255  N  V  L  K  K  I  D  L  P  Y  V  P  H  G  E  C  Q  T  A  L
901  CGCACGACCAGGCTCGGCGGATTCTTCAAGCTGGACAAGTCCTTCCTCTGCGCCGGAGGA
275  R  T  T  R  L  G  G  F  F  K  L  D  K  S  F  L  C  A  G  G
961  GAGGCTGGTAAGGACTCCTGCAGCGGCGACGGAGGTTCTCCTCTGGTTTGCCTGGACGCT
295  E  A  G  K  D  S  C  S  G  D  G  G  S  P  L  V  C  L  D  A
1021 ACCAAGACCCAGTACGTGCAGGTGGGCATTGTGGCGTGGGGCATCGGCTGCGGCACCTCC
315  T  K  T  Q  Y  V  Q  V  G  I  V  A  W  G  I  G  C  G  T  S
1081 AACATCCCTGGCGTGTACGCTGACGTGCTATACGGTTACGACTGGATCGTCGCTGAAGCT
335  N  I  P  G  V  Y  A  D  V  L  Y  G  Y  D  W  I  V  A  E  A
1141 GACAAGCTGCTTGCCAGTCCCGTGGTGGACTACTGGGGCTACGATTGAGCTGTGCAGGCG
355  D  K  L  L  A  S  P  V  V  D  Y  W  G  Y  D  *
1201 TCCCCACGGCCACACGCTGTCCCTCAAATCAAATGTAATATAAACTGCGATAATTAAATA
1261 AGTTAAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide (above) and deduced amino acid (below) sequences of a cloned *Sp*-SPH cDNA from crab *S. paramamosain*. Partial amino acid sequences of the different bands determined chemically were in bold and italic. The start codon, stop codon and putative polyadenylation signal (AATATA) were in bold and underlined. Cysteine residues forming the clip domain were in circles. The sites of catalytic triad of serine proteinase were in italic, bold and underlined. An arrow indicated the putative cleavage site for the signal peptide.

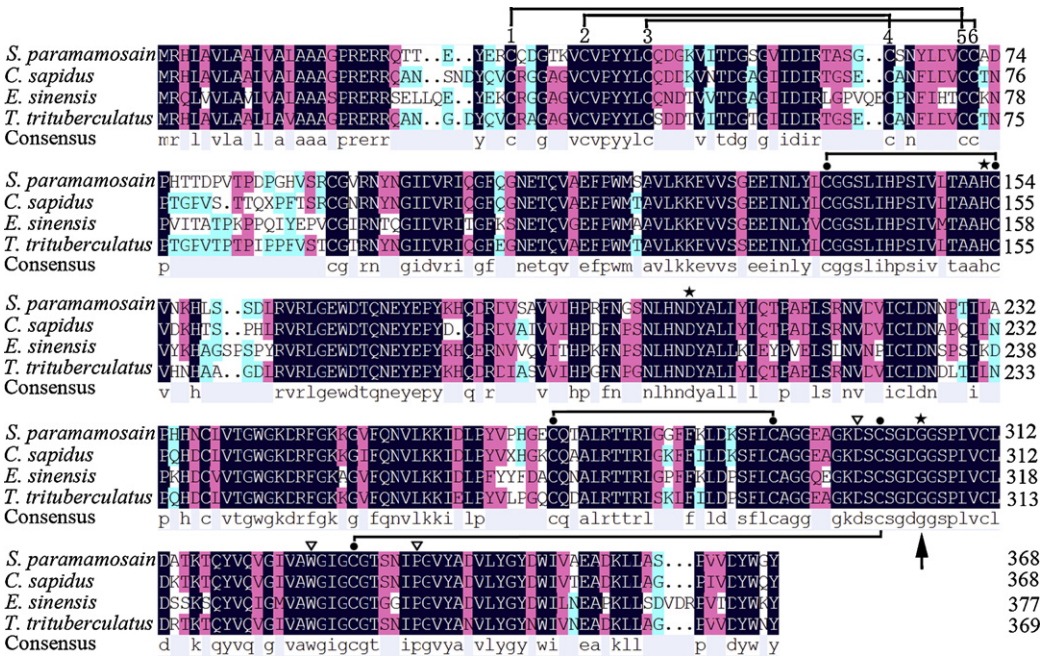

Fig. 3. Multiple alignments of crab *Sp*-SPH with known non-catalytic SPHs from other crab species, *C. sapidus*-PAF (AAS60227), *E. sinensis*-PAF (ACU65942), and *P. trituberculatus*-PAF (ACN87221). The six conserved cysteines in the clip domain were numbered and the linkages were indicated as Cys1-Cys5, Cys2-Cys4, and Cys3-Cys6. Gaps (–) were introduced to maximize the alignment of the clip domain cysteines. The catalytic triad of serine proteinase was indicated by stars; the amino acid residues forming the substrate specificity pocket in catalytically active serine protease were shown by open triangles; the conserved cysteine residues were shown in solid diamonds and three pairs of conserved cysteines forming putative intramolecular disulfide bonds were connected; the arrow indicated the substitution residue of the catalytic triad of serine proteinase. Gaps (–) were introduced to get maximal sequence similarity.

of *Sp*-SPH contained 1298 bp including 78 bp in the 5′ untranslated region (UTR), an open reading frame (ORF) of 1107 bp, and a 110 bp in the 3′-UTR with a polyA signal. The ORF of *Sp*-SPH cDNA included 369 amino acid (aa) residues with a 16 aa signal peptide predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) in the N-terminus. The calculated molecular mass of the mature protein without signal peptide was 38.7 kDa with an estimated isoelectric point of 5.46. The full-length cDNA sequence of *Sp*-SPH and its deduced aa sequence were deposited in the Genbank under accession number of GU250783. Blast analysis of amino acid sequences revealed that was similar to the SPH from other crab species including *C. sapidus* (AAS60227), *E. sinesis* (ACU65942), and *P. trituberculatus* (ACN87221). The putative protein sequence of *Sp*-SPH contained one N-terminal clip domain and a serine proteinase-like C-terminal domain. Sequences alignment analysis showed that the deduced *Sp*-SPH aa residues possessed high identity (83.8%) to those of aa residues from prophenoloxidase activating factor (PPAF) of *C. sapidus* (AAS60227), *E. sinesis* (ACU65942), and *P. trituberculatus* (ACN87221) (Fig. 3).

3.4. Tissue distribution of Sp-SPH mRNA transcripts in the crab

The distribution of *Sp*-SPH mRNA in different tissues was examined and the mRNA expression level of *Sp*-SPH varied among the samples tested. As shown in Fig. 4, the *Sp*-SPH mRNA transcripts were highly expressed in eye stalk, subcuticular epidermis, gill, hemocyte, and stomach, but were less expressed in thorax ganglion, brain and muscle according to the real-time PCR analysis. It was barely to detect the *Sp*-SPH expression in hepatopancreas, mid-gut, reproductive tract and heart. Thus, the variation of *Sp*-SPH expression suggested that the immune responses recruiting *Sp*-SPH was tissue specific in *S. paramamosain*. Further, we determined the mRNA expression of *Sp*-SPH in certain different development stages. Interestingly, the *Sp*-SPH existed in all detected stages and the highest expression was observed in the hemocytes of juvenile crabs followed by zoea, megalopa, and embryos (Fig. 5A). Statistically significant difference of *Sp*-SPH gene expression ($P < 0.01$) was observed in juvenile crabs when compared to that of embryo I stage. Whereas, the *Sp*-SPH transcripts were relatively lower in adult crabs compared to that of juvenile crabs and the zoea stage during development, suggesting it might play crucial roles in juveniles as well as other development stages. Based on the studies that SPH-containing proteins function as cofactors involved in proPO activation, we hypothesized that the *Sp*-SPH might also act in PO activation, we then examined if there was proPO transcript in the different development stages. As shown in Fig. 5B, the *Sp*-proPO transcripts were highly present in embryo (I, II, III and V) stage, zoea (I), juvenile and adult, but lower in megalopa. This result indicated that PO might play a role in the detected stages. But the role

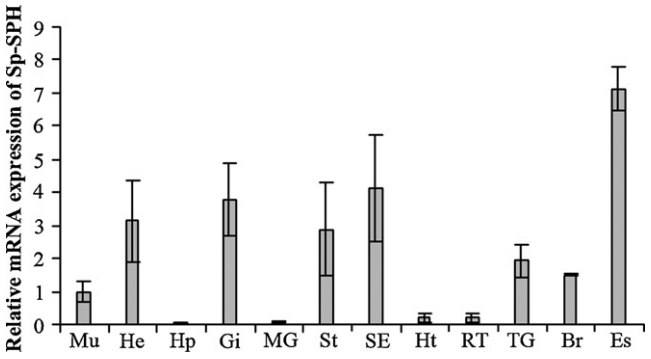

Fig. 4. Distribution of *Sp*-SPH mRNA transcripts in different tissues of crab *S. paramamosain*. The total RNA of different tissues was prepared as mentioned in Section 2 for quantitative real-time PCR. The relative gene expression was analyzed to the *Sp*-SPH mRNA expression in the muscle of the crab. Mu: muscle; He: hemocyte; Hp: hepatopancreas; Gi: gill; MG: mid-gut; St: stomach; SE: subcuticular epidermis; Ht: heart; RT: reproductive tract; TG: thorax ganglion; Br: brain; Es: eye stalk. The experiment was repeated for three times, and the data represented means of triplicates. Bars indicated mean ± S.E. ($n = 3$).

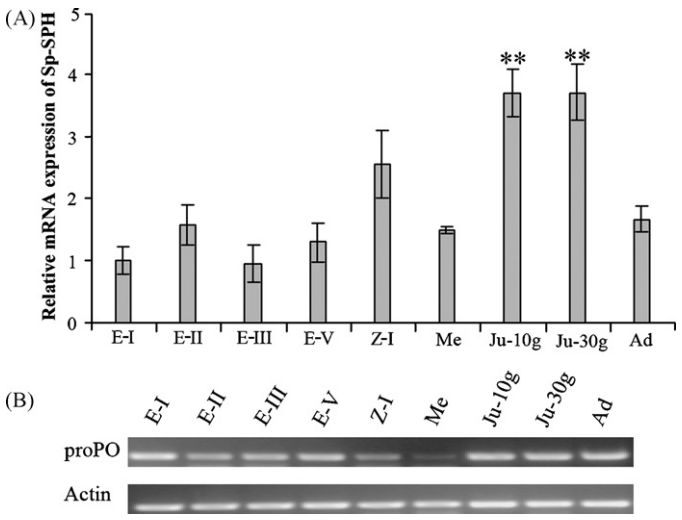

Fig. 5. (A) Determination of *Sp*-SPH transcripts in different development stages of crab *S. paramamosain*. The total RNA was prepared from different stages for quantitative real-time PCR as described above. The relative *Sp*-SPH mRNA expression was compared to that of embryo I stage. This experiment was performed for three times and the data demonstrated means of triplicates. Bars indicated mean ± S.E. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$. (B) Determination of *Sp*-proPO transcripts by semi-quantitative RT-PCR in different development stages of crab *S. paramamosain*. The cDNA prepared above was used for RT-PCR determination. The *Sp*-actin was employed as an internal control. This experiment was performed for three times. E-I: embryo stage I; E-II: embryo stage II; E-III: embryo stage III; E-V: embryo stage V; Z-I: zoea I stage; Me: megalops; Ju-10 g: hemocytes from juvenile crab (10 ± 2 g); Ju-30 g: hemocytes from juvenile crab (30 ± 5 g); Ad: hemocytes from adult crab.

of *Sp*-SPH related to proPO activation remains unknown and needs further study.

3.5. The mRNA expression profile of Sp-SPH against challenge of V. parahaemolyticus in hemocytes, subcuticular epidermis and gill using quantitative real-time PCR

The *Sp*-SPH was isolated by its binding activity to the crab pathogenic bacterium *V. parahaemolyticus*. To reveal the immune responses involving *Sp*-SPH against bacterial infection, we determined the mRNA expression profile of *Sp*-SPH post-challenge of *V. parahaemolyticus* using real-time PCR. Because the *Sp*-SPH mRNA transcripts showed high expression in the frontline immune defense tissues like hemocytes, subcuticular epidermis and gill of the crab, the differential expression pattern of *Sp*-SPH in these tissues during the time course of *Vibrio* challenge was analyzed. After bacterial challenge, the *Sp*-SPH gene expression was significantly increased in both hemocytes (3.3-fold, $P < 0.05$) and subcuticular epidermis (2.6-fold, $P < 0.05$) at 24 h and maintained relatively high expression till the end of the experiment at 96 h compared to that of saline injected crabs (Fig. 6A and B). In gill, the *Sp*-SPH transcript showed up-regulation at 48 h, and significant increase (2.8-fold, $P < 0.05$) occurred at 96 h post-challenge of *V. parahaemolyticus* (Fig. 6C). In contrast, no inducible mRNA expression of *Sp*-SPH in hepatopancreas was observed for both control and bacterial challenged animals (data not shown). These data together suggested that *Sp*-SPH gene had different expression profiles in the frontline defense tissues like hemocyte, subcuticular epidemis and gills post the challenge of a crab pathogenic bacterium.

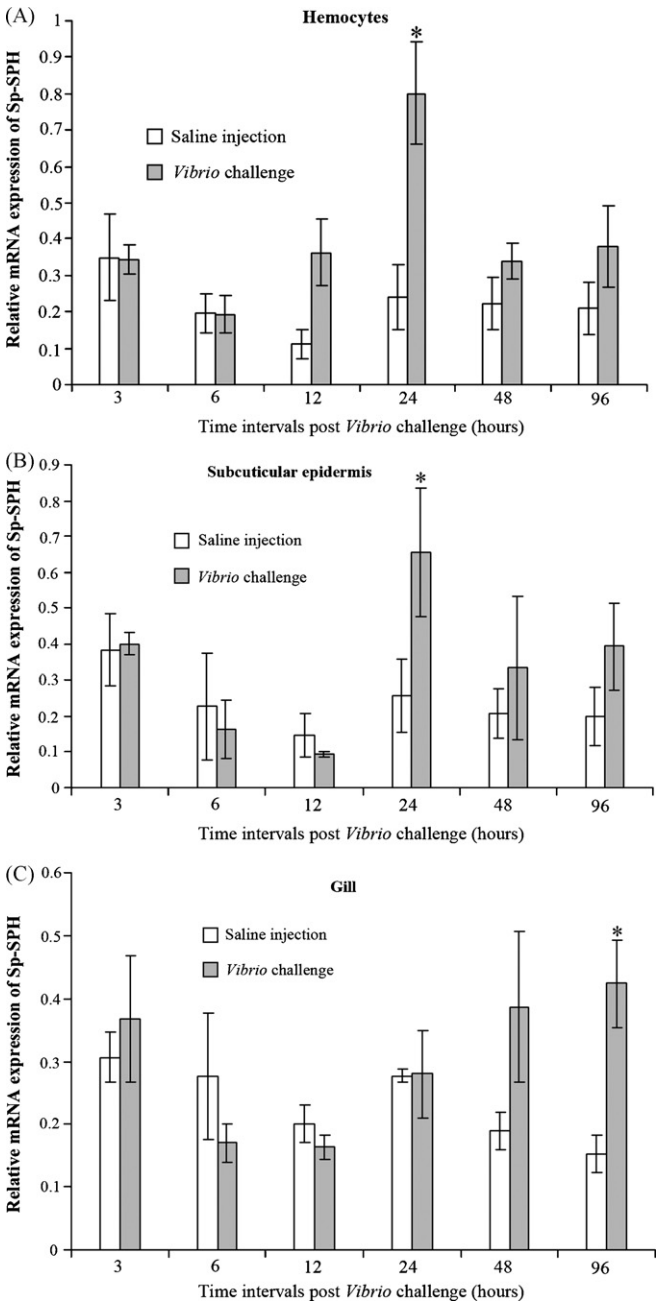

Fig. 6. The transcript expression profile of *Sp*-SPH against challenge of *V. parahaemolyticus* in hemocyte (A), subcuticular epidermis (B), and gill (C) of *S. paramamosain*. The relative mRNA expression of *Sp*-SPH was analyzed to its presence in crabs challenged by *V. parahaemolyticus* compared to those of saline injected animals. These data represented means of triplicates. Bars indicated mean ± S.E. ($n = 3$). $^{*}P < 0.05$.

4. Discussion

The isolated proteins were obtained by their binding activities to a live crab pathogenic bacterium—*V. parahaemolyticus*, and the *Sp*-SPH exhibited several proteolytic fragments (three bands ranging from about 34 to 45 kDa) on SDS-PAGE under reducing conditions. Amino acid sequences of these three different bands revealed that all of them were SPH-like proteins similar to that of *C. sapidus* [32]. Similarly, it has been reported in the freshwater crayfish *Pacifastacus leniusculus* that a masquerade like protein (also a SPH-containing protein) was processed to yield at least four subunits under reducing conditions after its binding to bacteria or yeast cell walls [25]. The important finding in our study was that, at least two molecules, *Sp*-SPH and *Sp*-ALF showed direct binding activity to a crab pathogenic bacterium *V. parahaemolyticus*. This result implied that both the AMPs and a putative melanization pathway might work together in the bacterial killing and elimination in

the crab. In contrast, no protein was isolated by bacteria-plasma binding preparation in the present study (data not shown). It is likely that the hemocytes contain the important immune factors like PRPs and AMPs present at biological boundaries prone to infection, where they provide an rapid and non-specific defense toward potentially intruding microorganisms [41]. Hence, this *Sp*-SPH protein might be present in hemocytes in a biologically inactive form and a proteolytic enzyme might cleave the intact *Sp*-SPH at certain specific sites. However, the details for the protein cleavage remain unclear and need further investigations.

The SPH proteins containing non-catalytic clip domain have been characterized from both vertebrates and invertebrates. After proteolytic cleavage, these SPH proteins will function in the immune responses of both vertebrates and invertebrates albeit the SPH-containing proteins do not possess proteinase enzyme activity. In arthropods, the clip domain was shown to act in protein–protein interactions, regulation of the proteinase activity, and bactericidal activities [42]. So far, many SPH-containing proteins have been identified from various species and they possess inactive serine proteinase-like domains exhibiting biologically important roles, such as proPO activation [10,43], cell adhesion activity [24,26], LPS binding activity [44] and antimicrobial activity [45–47]. A crayfish SPH-containing protein (a masquerade like protein) was suggested to possess opsonic activity against *E. coli* [25]. Because the crab HLS treated *V. parahaemolyticus* was coated with both *Sp*-SPH and *Sp*-ALF proteins, we could not use these coated bacteria to perform opsonic experiments, and further study using recombinant *Sp*-SPH is then necessary to elucidate if crab *Sp*-SPH also works as an opsonic factor like that in crayfish.

The *Sp*-SPH mRNA transcript was present in various tissues in the normal crab as determined by real-time PCR, and it was abundant in the frontline defense tissues such as gill, subcuticular epidermis and hemocytes. In shrimp, a SPH gene also showed strong expression in hemocyte and gill [27]. However, it was barely to be detected in the hepatopancreas of both crab and shrimp. This was distinct from that of *Es*SPH gene which showed the highest expression in the hepatopancreas of Chinese mitten crab *E. sinesis* [33]. Besides, the eye stalks contain many physiological and immune regulatory factors like hormones in arthropods [48]. A higher *Sp*-SPH mRNA expression existed in the crab eye stalks, suggesting this protein might play certain roles in the physiological processes and immune regulations which needs further studies to be elucidated. A relative higher transcript expression of *Sp*-SPH was also observed from embryo to juvenile crab. The variation of SPH gene expression in different organs/development stages and various species indicates the immune reaction related to SPH is tissue/development stages and species specific in crustaceans. Importantly, the crabs need to molt periodically during their development process including larvae to juvenile stages. For example, 6–7 ecdysis occurs from zoea stage to megalopa stage. They usually show high mortality rate during these stages which are much more sensitive to pathogenic infections. Accordingly, a relatively lower proPO transcript was detected in the megalopa stage by RT-PCR, implying that PO activity might play a role in the protection during these stages. Besides, the proPO transcripts were present in embryo stages (I, II, III and V) but lower in megalopa, and then showed high expression again in juveniles, implying that proPO might be maternal transferred as suggested by Qin et al. [33]. However, this concept still needs further investigations. In contrast, very low or no proPO transcript was present in the nauplius-4 stage in shrimp, and the low proPO expression might be from a very low number of hemocytes but not mother transferred as the authors suggested [49]. The PO activity test is needed to elucidate the role of proPO-system in the embryonic stages of crab in future studies. Further, no proPO transcript was detected from the embryo of freshwater crayfish in a latest report [50]. These different profiles of proPO transcript in the developing stages might be due to species specific features among crab, shrimp and crayfish, which is not clear yet and necessary to be further investigated. These data together implied that the *Sp*-SPH and putative melanization might be important for both immune defense and cuticle sclerotization or tanning during development stages of the crabs.

The hemocyte, subcuticular epidermis and gill are the frontline organs facing against invading microorganisms. All of these tissues are able to generate melanin to inhibit or kill the invading bacteria. After challenge with crab pathogenic *V. parahaemolyticus*, a significant increased mRNA expression of *Sp*-SPH occurred in both hemocytes and subcuticular epidermis at 24 h and remained relatively high expression till 96 h. Meanwhile, the strong up-regulation of *Sp*-SPH transcript was also shown up in the gill at 96 h post bacterial challenge. These results demonstrated that the *Sp*-SPH gene serves as an acute-phase defense molecule and might play crucial roles in frontline immune defense tissues for antibacterial responses in crab.

In conclusion, our data together suggest clearly for the first time that a *Sp*-SPH protein is involved in the recognition and defense of invading bacteria in the crab *S. paramamosain*. Further studies are still necessary for elucidating its binding specificity to different microorganism like Gram-positive bacteria, more other Gram-negative bacteria, yeast and fungi. The elucidation of the possible role of *Sp*-SPH in opsonic regulation and the activation of immune response cascade like proPO activation is also wanted. These investigations will benefit for revealing the antibacterial mechanism in the crab and generating useful biological information for prevention and control of crab disease, as well as selection of fine genetic breeding in crab farming.

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