Three Kazal-type serine proteinase inhibitors from the red swamp crayfish
Procambarus clarkii and the characterization, function analysis of hcPcSPI2

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ABSTRACT
Three Kazal-type serine proteinase inhibitors, hcPcSPI2, hpPcSPI3, and hpPcSPI4, with complete cDNA sequences, were identified from a cDNA library of the red swamp crayfish, Procambarus clarkii. Semi-quantitative RT-PCR shows that hcPcSPI2 exists mainly in hemocytes while both hpPcSPI3 and hpPcSPI4 were detected in the hepatopancreas and the heart. Homology comparison and phylogenetic analysis indicate that hpPcSPI3 and hpPcSPI4 shared high identity and formed the same group, and both of them were different from other hepatopancreas type inhibitors in crustaceans forming a large group, while hcPcSPI2 as well as other hemocyte type inhibitors belonged to another cluster. In addition, the temporal expression profiles of these three inhibitors were studied with quantitative real-time PCR and the results suggest that hcPcSPI2 and hpPcSPI3 are likely to be involved in antiviral immune response, and all these three inhibitors respond to Vibrio anguillarum challenge in different degrees. Further study was done on hcPcSPI2. Western blot demonstrates that hcPcSPI2 only exists in semigranular cells. Besides, after V. anguillarum challenge, the hcPcSPI2 protein could also be detected in cell-free hemolymph. Subsequently, the biochemical characteristics and bacteriostatic activity of hcPcSPI2 were assayed. The results indicate that hcPcSPI2 shows weak inhibitory activity against subtilisin A and trypsin, and may trigger bacteriostatic activity towards Bacillus subtilis and Bacillus thuringiensis, possessing MIC90 of 30.4 and 25.0 μM, respectively. These studies reveal that hcPcSPI2 may also play an important role in the antibacterial immunity of the crayfish.

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1. Introduction
Kazal-type serine proteinase inhibitors (SPIs) are known to inhibit serine proteinases and are widely distributed in most of organisms. In recent years, numerous new members of the Kazal-type inhibitor family were identified as having various physiological functions. For example, a human inhibitor, LEKTI, may play a part in anti-inflammatory protection [1]. A male reproduction-related SPI isolated from Macrobachium rosenbergii also possesses inhibitory effects on sperm gelatinolytic activity [2]. BmSPI from Bombyx mori might also function as an inhibitor to the microbial proteinases and protect the silkworm pupae from infection by pathogens [3]. Kazal2 from hydra also show potent bactericidal activity against Staphylococcus aureus in vitro [4]. In addition, several parasites and microbial pathogens have been known to generate SPIs to counter the protective proteinases of hosts. For instance, the SPI produced by Toxoplasma gondii can protect the parasite from the digestive enzymes in host intestines [5]. Rhodnius prolixu and Triatoma infestans can secrete potent thrombin inhibitors, Rhodnin and Infestin, respectively, in order to prevent blood coagulation and make it easier to suck blood from their hosts [6,7]. In addition, the oomycete Phytophthora infestans produces an extracellular protease inhibitor to counter the defensive proteinases of hosts [8,9].

Recently, a variety of Kazal-type SPIs were found in crustaceans [10,11]. Several of these inhibitors are believed to be involved in innate immune response. For example, the two-domain and seven-domain Kazal-type SPIs from the Chinese white shrimp Fenneropenaeus chinensis were found to participate in anti-WSSV and anti-bacterial immune response, respectively [12,13]. In addition, the inhibitors SPI1n2 from the black tiger shrimp Penaeus monodon and hcPcSPI1 from the crayfish Procambarus clarkii, both showing inhibitory activity against subtilisin, are believed to defend against invading bacterial pathogens [14,15].
Kazal-type SPIs usually contain more than one Kazal-domain. The typical domain is characterized by the presence of six well-conserved cysteine residues forming three intra-domain disulfide bridges (1–5/2–4/3–6), which are responsible for the stability of the structure. The reactive center on the surface of the inhibitor consists of a segment with a convex and solvent-exposed loop, which is highly complementary to the concave active site of proteinases [16,17]. The P1 residue, which is believed to contribute mainly to the inhibitory specificity, is located at the second position after the second cysteine residue of the domain [18], which is the most exposed position of the reactive center. Recent studies report that Kazal-type inhibitors react with cognate enzymes based on a substrate-like standard mechanism, which is in good agreement with the view of Laskowski [19].

In our laboratory, a cDNA library was constructed using the hepatopancreas and the gills of P. clarkii. From the library, four Kazal-type SPIs with full-length cDNA sequences were identified. One of the SPIs, hcPcSPI1, has been reported recently [15]. In this study, the tissue distributions of the other SPIs were examined using semi-quantitative RT-PCR. Taking into account the fact that these three SPIs were identified from WSSV-infected crayfish, we first investigated the expression profiles with quantitative real-time PCR (qRT-PCR) to evaluate whether they were involved in antiviral immunity, and then the expression patterns after Vibrio anguillarum challenge were further examined. In addition, hcPcSPI2 was chosen for further study. The expression profiles at protein level and hemocyte type distribution were detected using the prepared antibody against hcPcSPI2. Subsequently, hcPcSPI2 was overexpressed and purified to assay its biochemical characteristics and bacteriostatic activities. The results reveal that all these three inhibitors respond to V. anguillarum challenge in different degrees, and hcPcSPI2 and hpPcSPI3 may also play a part in the antiviral immune response of crayfish. Besides, it is possible that hcPcSPI2 defends against invading bacterial pathogenes directly.

2. Materials and methods

2.1. Challenge of crayfish and collection of tissues

Red swamp crayfish P. clarkii, were purchased from a market in Jinao, Shandong Province, China and temporarily kept in air-pumped water at room temperature. To investigate the temporal expression profiles after pathogen challenge, the crayfish were categorized into two groups randomly. For the challenge group, each normal healthy crayfish (107 cells) or 50 μl of infected tissue homogenate containing 105 copies) from the abdominal segment. The control was treated (RNase-free DNase I, Takara, Japan) total RNA (5 μg) was reverse transcribed into first-strand cDNA, which was used as the templates for the following semi-quantitative RT-PCR and qRT-PCR after dilution in nuclease-free water.

2.5. Semi-quantitative RT-PCR and qRT-PCR

Tissue distribution was analyzed using semi-quantitative RT-PCR. The primers, F2 and R2 for hcPcSPI2, F3 and R3 for hpPcSPI3, and F4 and R4 for hpPcSPI4 (Table 1), were designed to generate the corresponding specific target fragments respectively. In addition, the primers SF and SR (Table 1) were used to amplify 18sRNA as the reference. The amplification profile consisted of the initial step of 94 °C for 3 min followed by 25 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 20 s, and then 72 °C for 10 min.

Table 1

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibitors</strong></td>
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</tr>
<tr>
<td>F2</td>
<td>GTGGGAAAGTTATGCTTGAGG</td>
</tr>
<tr>
<td>R2</td>
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<td>F3</td>
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<td>F4</td>
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<td>R4</td>
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<tr>
<td>ER2</td>
<td>TACTCATGCGT</td>
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<tr>
<td><strong>18sRNA</strong></td>
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<tr>
<td>SR1</td>
<td>AAGGGGATGAACGGGTT</td>
</tr>
</tbody>
</table>

The underlined nucleotides indicate the locations of restricted endonucleases (EcoRI or XhoI).
To investigate the temporal expression profiles of inhibitors after pathogen challenge, qRT-PCR was performed following the protocol previously described [23]. Three pairs of primers (F2, R2; F3, R3; and F4, R4) for inhibitors and one pair of primers SF1 and SR1 (Table 1) for the reference 18sRNA were designed for quantitative analysis. The qRT-PCR was programmed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 59 °C for 25 s, 72 °C for 20 s, and then melting from 65 to 95 °C for final product analysis. All tests were repeated at least three times using individual templates. The data obtained were treated with statistical analysis and calculated by 2-ΔΔct followed by an unpaired sample t-test. A significant difference was accepted at P < 0.05.

2.6. Recombinant expression, purification, and antisera preparation of hcPcSPI2

According to the full-length cDNA sequence of hcPcSPI2, two expression primers (EF2, ER2) were designed to amplify the sequence encoding the corresponding mature peptide (Table 1). The specific fragment was digested by EcoR I and Xho I and then inserted into a pET-30a (+) vector. The expression vector of hcPcSPI2 was then transformed into competent Escherichia coli Rosetta (DE3) host. IPTG was added to a final concentration of 0.5 mM to induce protein expression at 28 °C for 10 h. The purification and antisera preparation of hcPcSPI2 were performed similar to the methods described previously [24].

2.7. Western blot analysis of tissue distribution and expression profiles of hcPcSPI2

The cell-free hemolymph, hemocytes, heart, hepatopancreas, gills, stomach, and intestine of normal crayfish were homogenized in a buffer (50 mM Tris—HCl, pH 7.5, 150 mM NaCl, 3 mM EDTA and 1 mM PMSF) and then centrifuged at 10,000 × g for 15 min to collect the supernatant. Also, hemocytes separation from normal crayfish on preformed Percoll gradients according to the previous method [20] was done to further examine in which type of hemocytes hcPcSPI2 existed. In addition, the cell-free hemolymph and hemocytes of V. anguillarum-challenged crayfish were prepared at 12, 24, and 48 h post-infection. The protein concentration was assayed following the method of Bradford [25]. Each sample (60 μg protein) was analyzed on 12.5% SDS-PAGE according to the Laemmli method [26]. After that, the proteins were transferred onto a nitrocellulose membrane. The membrane was first blocked with 3% non-fat milk in PBS (10 mM Tris—HCl, pH 7.5, 150 mM NaCl) for an hour, then incubated with the 1/100 diluted antisera to hcPcSPI2 overnight, and finally visualized by a colorimetric reaction catalyzed by peroxidase-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS).

2.8. Inhibitory assays of rhcPcSPI2

Five kinds of serine proteinases, namely, trypsin (bovine pancreas, Sigma), chymotrypsin (typelobovine pancreas, Sigma), elastase (Porcine pancreas, Sigma), subtilisin A (Bacillus licheniformis, Sigma) and proteinase K (Trichthium album, Merck), were used to assay the inhibitory activities of hcPcSPI2 according to previously reported methods [13]. In brief, in addition to the proteinase, the components (Table 3) were composed of the corresponding chromogenic substrate (Nα-benzoyl-d,L-arginine-p-nitroanilide (BAPNA) (Sigma) for trypsin; N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (AAPF) (Sigma) for α-chymotrypsin, subtilisin and proteinase K; N-succinyl-Ala-Ala-Ala-p-nitroanilide (SAAAN) (Sigma) for elastase) and rhcPcSPI2 with a different concentration in a total volume of 100 μl reaction buffer (100 mM Tris—HCl, pH 8.0). The reaction was incubated at 30 °C for 5 min (15 min for trypsin) and then terminated with 50% acetic acid.

2.9. Kinetics of proteinase inhibition

This experiment consisted of four groups of reactions, to which four different concentrations of recombinant protein hcPcSPI2 were applied. Each group was comprised of four different concentrations of artificial chromogenic substrate while the amounts of proteinase and inhibitor were fixed. For subtilisin inhibition, 0.15, 0.20, 0.30, and 0.40 mM of AAPF were incubated with 3.67 nM of subtilisin and a fixed amount of hcPcSPI2. Subsequently, four different concentrations of hcPcSPI2: 0, 0.12, 0.24, and 0.36 μM were applied. For trypsin inhibition, 0.5, 0.75, 0.10, and 1.50 mM of BAPNA were incubated with 0.40 μM of trypsin and a given concentration of hcPcSPI2. Next, four different concentrations of hcPcSPI2: 0, 12, 24, and 36 μM, were used. The procedure of mixing the components was carried out following the steps described above. The Ks and Vmax of proteinases and the inhibition constants Ks of rhcPcSPI2 were calculated based on the former methods [5,27].

2.10. Bacteriostatic assay

Bacteriostatic assays of rhcPcSPI2 were performed on the following, based on the procedure previously described [15,28,29]: microorganisms containing three kinds of Gram-negative bacteria, namely, V. anguillarum, E. coli and Klebsiella pneumoniae, microorganisms containing five kinds of Gram-positive bacteria, namely, Bacillus subtilis, Bacillus megaterium, Bacillus cereus, Bacillus thuringiensis and S. aureus and one species of fungi, Saccharomyces cerevisiae. The recombinant protein rhcPcSPI2 was subjected to twofold serial dilution in PBS in a plain 96-well plate. Both the BSA and BSA-pla4 protein (the latter was expressed with the same expression vector) were also diluted and used as the negative control.

3. Results

3.1. cDNAs cloning

From the cDNA library of red swamp crayfish, P. clarkii, three Kazal-type serine proteinase inhibitors with full-length cDNA sequences were identified and designated as hcPcSPI2, hpPcSPI3, and hpPcSPI4, respectively. Detailed information regarding these three sequences is listed in Table 2. The complete cDNA sequence of hcPcSPI2 had 1195 bp with an open reading frame (ORF) of 402 bp encoding a 133 amino acids protein, a 5′ untranslated region (UTR) of 75 bp, a 3′ UTR of 718 bp with a canonical polyadenylation signal sequence AATAAA, and a poly (A) tail. The deduced protein sequence consisted of a putative 22 aa signal peptide and two tandem Kazal domains. In addition, there were two potential protein kinase C

Table 2

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Accession number</th>
<th>Full-length of cDNA</th>
<th>Number of AA</th>
<th>Signal peptide</th>
<th>Numbers of domains</th>
<th>P1 residues</th>
</tr>
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<tbody>
<tr>
<td>hcPcSPI2</td>
<td>GU062747</td>
<td>1195 bp</td>
<td>133</td>
<td>MAPRATILLHAVMLVVLVALAQG</td>
<td>2</td>
<td>R; S</td>
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<tr>
<td>hpPcSPI3</td>
<td>GU062748</td>
<td>972 bp</td>
<td>133</td>
<td>MSIANLVLFSGLIGAGS</td>
<td>2</td>
<td>T; T</td>
</tr>
<tr>
<td>hpPcSPI4</td>
<td>GU062749</td>
<td>1091 bp</td>
<td>180</td>
<td>MSIRAGLIFSLALARC</td>
<td>3</td>
<td>D; D; T</td>
</tr>
</tbody>
</table>
phosphorylation sites located between the two domains. The hcPcSPI2 protein had a theoretical molecular weight (Mw) of 14.2 kDa and an isoelectric point (pI) of 8.50.

The complete cDNA sequences of hpPcSPI3 and hpPcSPI4 were 972 bp with an ORF of 402 bp encoding 133 aa and 1091 bp with an ORF of 543 bp encoding 180 aa, respectively. Both also contained signal peptides, 5' and 3' UTRs, and poly (A) tails. The theoretical Mw/pI was 14.3/4.02 for hpPcSPI3, and 19.8/3.70 for hpPcSPI4.

All above sequences were deposited in GenBank with the following accession numbers: GU062747 (hcPcSPI2), GU062748 (hpPcSPI3), and GU062749 (hpPcSPI4).

3.2. Homology and phylogenetic tree analysis

The BLASTP search indicates that hcPcSPI2 shared the highest similarity with the semigranular hemocyte specific Kazal-type proteinase inhibitor (75% identity, ACB78013) from crayfish Pacifastacus leniusculus, hpPcSPI3 showed the highest identity with FcSPI-1 (39%, AAP92780) derived from the hepatopancreas of the Chinese white shrimp, and hpPcSPI4 shared the highest identity with the hepatopancreas type proteinase inhibitor (51%, ACL36280) from the black tiger shrimp. Besides, it was found that hpPcSPI3 and hpPcSPI4 showed 56.67% identity with each other through homology comparison. According to the BLASTP results, multiple alignments of domains of four Kazal-type inhibitors from red swamp crayfish and others also from crustaceans were performed and analyzed (Fig. 1). The results indicate that except for the second domain of hpPcSPI3 (hpPcSPI3D2) and the third domain of hpPcSPI4 (hpPcSPI4D3), both of which showed higher identity,
all the other domains of these four inhibitors had the conserved motif of C1x3C2x5PVC3Gx4TYx3C4xLx4C5x10e12GxC6. Moreover, for the above mentioned two domains (hpPcSPI3D2 and hpPcSPI4D3), there are more residues between C1 and C2, as well as the C5 and C6 than the other domains (Fig. 1). Besides, only two amino acid residues exist between the C4 and C5 of the two domains, while in the others the number is six or seven. These differences may suggest that hpPcSPI3 and 4 are two novel inhibitors with different physiological functions.

Based on the BLASTP results, a phylogenetic tree was constructed using these four inhibitors from P. clarkii and other Kazal-type inhibitors from crustaceans to study the evolutionary relationship among them. As the phylogenetic tree illustrated in Fig. 2, all the inhibitors from hepatopancreas were grouped into a large cluster except hpPcSPI3 and 4, which formed a small group, while all the other inhibitors from hemocytes including hcPcSPI1 and hcPcSPI2 belonged to another large group.

3.3. Tissue distribution and expression pattern analysis

The tissue distribution of these three Kazal-type inhibitors was detected using semi-quantitative RT-PCR. The results showed that hpPcSPI2 was highly expressed in hemocytes but also could be detected in the heart, gills, intestine, and stomach of normal crayfish, while hpPcSPI3 and hpPcSPI4 were mainly distributed in the hepatopancreas and heart (Fig. 3).

Quantitative RT-PCR was used to analyze the temporal expression profiles after pathogen challenge. After WSSV injection, hpPcSPI2 increased gradually, reaching the highest level (~15-fold increase) at 72 h post-challenge, after which it decreased (Fig. 4A). As illustrated in Fig. 4C, the transcripts of hpPcSPI3 in the hepatopancreas first increased and obtained the highest level by 24 h and then gradually dropped down after WSSV challenge, while the transcripts of hpPcSPI4 did not show any obvious change post-WSSV challenge (Fig. 4E).

When challenged with V. anguillarum, the transcripts of hcPcSPI2 considerably ascended and reached the top level (~9-fold increase) at 12 h post-challenge, and then gradually decreased (Fig. 4B). For hpPcSPI3 and hpPcSPI4, they both upregulated and gained their respective top levels (~1.5-fold increase for each) at 12 h after V. anguillarum challenge, and then came back to the normal expression level (Fig. 4D, F).
3.4. Recombinant expression and purification of hcPcSPI2

The recombinant plasmid was transformed into *E. coli* Rosetta (DE3) for protein expression. After induction with 0.5 mM IPTG, hcPcSPI2 was overexpressed as inclusion bodies. It was then purified by His-Bind Resin Chromatography after refolding. Given that the recombinant protein contained 52 additional amino acid residues (5.7 kDa) of the vector, the result of SDS-PAGE indicated that the size of rhcPcSPI2 was approximately consistent with the predicted molecular weight (Fig. 5).

3.5. Tissue and hemocyte type distribution, and expression profile of hcPcSPI2 at protein level

Western blot analysis was performed to investigate the tissue and hemocyte type distribution, and expression pattern of hcPcSPI2. The results indicate that, consistent with the semi-quantitative RT-PCR results, hcPcSPI2 was found in the hemocytes, heart, gills, intestine, and stomach but not in cell-free hemolymph or in the hepatopancreas in normal crayfish (Fig. 6A). Furthermore, it was found that hcPcSPI2 existed only in semigranular hemocytes, not in hyaline or granular hemocytes (Fig. 6B). Since the transcripts of hcPcSPI2 increased at 12 h and recovered at 48 h after *V. anguillarum* injection, we also detected the hcPcSPI2 protein in cell-free hemolymph and hemocytes at 0, 12, 24, and 48 h post-bacterial challenge. The results demonstrate that hcPcSPI2 protein decreased after 12 h in hemocytes (Fig. 6C) then gradually went back to normal, while it appeared in the cell-free hemolymph at 12, 24, and 48 h post-bacterial challenge (Fig. 6D).

3.6. Inhibition assays

The inhibitory activity of hcPcSPI2 was assayed towards trypsin, chymotrypsin, elastase, subtilisin A, and proteinase K. The results indicate that hcPcSPI2 could weakly inhibit subtilisin A and trypsin, but not chymotrypsin, elastase, and proteinase K (Fig. 7A). As
transcripts in the black tiger shrimp *P. monodon* and twelve transcripts in the crayfish *P. leniusculus* were reported recently [10–13]. Some of them were found to have important roles in the innate immunity of their respective organisms [12–15]. To date, four Kazal-type inhibitors with full-length sequences have been identified from WSSV-infected crayfish, and hcPcSPI1 has been reported [15]. In this paper, we will further study hcPcSPI2, hpPcSPI3, and hpPcSPI4.

From the phylogenetic tree, we can see that hpPcSPI3 and hpPcSPI4, both derived from the hepatopancreas and different from other hepatopancreas type inhibitors of shrimp, formed a small group. This indicates that hpPcSPI3 and hpPcSPI4 have a relatively distant evolutionary relationship with other inhibitors from hepatopancreas. Thus, it may suggest that these two inhibitors have different functions compared with the others from hepatopancreas.

In addition, the inhibitors from hemocytes seem hard to be grouped into one meaningful cluster since the Bootstrap values are very low, which reveals that these hemocyte type inhibitors, including hcPcSPI1 and hcPcSPI2, evolve rapidly. A similar result was also reported through homology comparison and phylogenetic analysis of more Kazal-type inhibitors from crayfish *P. leniusculus* and the black tiger shrimp [11]. Considering the abundance of Kazal-type inhibitors in crustacean hemocytes, the high variability may suggest that they are involved in many kinds of physiological processes.

Since these three inhibitors were identified from WSSV-challenged crayfish, the expression profiles post-WSSV injection were investigated to see if these inhibitors participated in antiviral immune response. The current study showed that hcPcSPI2 in hemocytes was upregulated after WSSV challenge, whereas our previous study demonstrated that hcPcSPI1 was downregulated [15]. Though both originated from hemocytes, they showed low identity. Taking into account that the inhibitors from hemocytes evolve rapidly, we speculate that hcPcSPI1 and hcPcSPI2 are two different kinds of Kazal-type inhibitors and may participate in anti-pathogenic immunity in two different ways.

Compared with the inhibitors from hemocytes in crustaceans, researches on Kazal-type inhibitors from the hepatopancreas are much less. One recent report demonstrates that the hepatopancreas type inhibitor *Fc*SPI-1 participates in the immune defense against *V. anguillarum*, but not WSSV [13]. Different from *Fc*SPI-1, hpPcSPI3 was upregulated after WSSV challenge in our study. Kazal-type inhibitors from the hepatopancreas are believed to be secreted into the plasma or gastrointestinal system to exert different physiological functions. This means that hpPcSPI3 may be released into the hemolymph and has a role in antiviral immunity. In addition, although hpPcSPI4 shared high similarity with hpPcSPI3, there was no obvious change for hpPcSPI4 after WSSV challenge. It is possible that hpPcSPI4 performs other physiological functions.

Though it was found that the transcripts of all these three inhibitors increased after *V. anguillarum* challenge, hcPcSPI2 seemed to be more sensitive to *V. anguillarum* than hpPcSPI3 and hpPcSPI4 since hcPcSPI2 was upregulated more greatly than the latter two. These findings reveal that hpPcSPI2 in hemocytes may play a more important role in defending against *V. anguillarum* invasion than hpPcSPI3 and hpPcSPI4 which both exist in hepatopancreas. In addition, at protein level there was no obvious change for the hcPcSPI2 in hemocytes except for a decrease at 12 h after *V. anguillarum* stimulation. Intriguingly, hcPcSPI2 was also detected in cell-free hemolymph after *V. anguillarum* challenge. Similar results were also found in the plasma of the crayfish that were injected with LPS, which is the main component of Gram-negative bacterial cell wall [30]. The above results reveal that after *V. anguillarum* challenge, the stored hcPcSPI2 was purposefully secreted into circulating hemolymph. Meanwhile, the transcript of hcPcSPI2 was induced, and the protein was synthesized to be recruited or further be released into the hemolymph. Since
our former study showed $hcPcSPI1$ was upregulated in response to another Gram-negative bacterial challenge (specifically $E. coli$)\cite{15}, it suggests that $hcPcSPI1$ and $hcPcSPI2$ might also participate in defending against Gram-negative bacteria.

Western blot shows that $hcPcSPI2$ exists in semigranular cells only. This result is similar with a marker protein residing in semigranular hemocytes from crayfish $P. leniusculus$\cite{30}. Since $hcPcSPI2$ have comparatively high identity with this marker protein, we believe that $hcPcSPI2$ is also a marker protein of semigranular cells in crayfish $P. clarkii$. In addition, the $hcPcSPI2$ was detected in heart and gills mainly because of these tissues always full of hemocytes, while the fact that it was found in stomach and intestine might be due to the contamination of hemocytes since the hemolymph in crayfish was ubiquitous. Anyway, the ubiquitousness in crayfish could facilitate $hcPcSPI2$ to function more effectively when necessary. Given its feasible functions in immune response, $hcPcSPI2$ might be more than a marker protein.

In our previous study $hcPcSPI1$ showed strong inhibitory activities against subtilisin $A$ and proteinase $K$\cite{15}. In this study, recombinant $hcPcSPI2$ were also applied to assay proteinase inhibitory activity. It is well known that the specificity of inhibition mainly depends on the $P1$ amino acids of the Kazal-type inhibitors. According to the previous study, inhibitors with $P1$ Lys and Arg always inhibit trypsin and trypsin-like proteinases, and those with $P1$ Ala and Ser are apt to inhibit elastase\cite{19}. By virtue of $hcPcSPI2$ with $P1$ Arg and Ser, proteinases including trypsin, chymotrypsin, subtilisin, proteinase $K$ and elastase were used to assay the inhibitory activity of $hcPcSPI2$. It was found that although previous study demonstrated the $P1$ Ser was responsible for elastase inhibition\cite{31}, $hcPcSPI2$ could only weakly inhibit trypsin and subtilisin, and could not inhibit elastase at all. It was then tempting to suppose that the Arg might contribute to trypsin inhibition; whether both Arg and Ser contributed to subtilisin inhibition needed further study. The clue was derived from the study of the following two Kazal-type inhibitors. The inhibitor with $P1$ Ser in Bobwhite quail ovomucoid and CmPI-II with $P1$ Arg from the marine snail $Cenchrusis muricatus$ both showed inhibitory activity against subtilisin\cite{31,32}.

Based on the inhibitory activity of $hcPcSPI2$ on subtilisin and trypsin, we further studied their kinetics. The $K_i$s of $hcPcSPI2$ against subtilisin and trypsin were 0.057 and 3.729 $\mu M$ respectively. As anticipated, the inhibition constant was higher compared with 0.52 nM against subtilisin of $hcPcSPI1$\cite{15}. We speculate that the target proteinase of $hcPcSPI2$ in the cell was different from the proteinases tested in this study, which might influence the final inhibitory activities.
Former reports showed that Kazal-type inhibitors with inhibitory activities towards subtilisin might possess bacteriostatic activity against some bacteria, particularly *B. subtilis* [14,33]. Our previous research showed that the weaker the subtilisin inhibition, the lower the bacteriostatic activity on *B. subtilis* [15]. Here, hcPcSPI2 showed weak inhibition on subtilisin, and could only inhibit the growth of *B. subtilis* at high concentration. This kind of phenomenon further confirms our former conclusion that inhibitors might influence the growth of microorganisms via inhibiting the subtilisin that they secrete.

**Table 4** Bacteriostatic activity of recombinant hcPcSPI2.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC₅₀ (µM)</th>
</tr>
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<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>30.4</td>
</tr>
<tr>
<td>Bacillus thuringiensis</td>
<td>25.0</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>&gt;35.0</td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>&gt;35.0</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>&gt;35.0</td>
</tr>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli (8099)</td>
<td>35.0</td>
</tr>
<tr>
<td>Vibrio anguillarum</td>
<td>35.0</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>&gt;35.0</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>&gt;35.0</td>
</tr>
</tbody>
</table>

Bacteriostatic activity was determined using a microdilution susceptibility assay. MIC₅₀, Minimum Inhibitory Concentration required to inhibit the growth of 50% of microorganisms; >, no inhibition found at the concentration indicated.

**Acknowledgements**

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**References**


