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A three-domain Kazal-type serine proteinase inhibitor exhibiting domain inhibitory and bacteriostatic activities from freshwater crayfish *Procambarus clarkii*

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ABSTRACT

In crustaceans, Kazal-type serine proteinase inhibitors in hemolymph are believed to function as regulators of the host-defense reactions or inhibitors against proteinases from microorganisms. In this study, we report a Kazal-type serine proteinase inhibitor, named hcPcSPI1, from freshwater crayfish (*Procambarus clarkii*). We found that hcPcSPI1 is composed of a putative signal peptide, an RGD motif, and three tandem Kazal-type domains with the domain P1 residues L, L and E, respectively. Mainly, hcPcSPI1 was detected in hemocytes as well as in the heart, gills, and intestine at both the mRNA and protein levels. Quantitative real-time PCR analysis showed that hcPcSPI1 in hemocytes was upregulated by the stimulation of *Escherichia coli* (8099) or became decreased after a white spot syndrome virus (WSSV) challenge. In addition, hcPcSPI1 and its three independent domains were overexpressed and purified to explore their potential functions. All four proteins inhibited subtilisin A and proteinase K, but not α-chymotypsin or trypsin. Recombinant hcPcSPI1 could firmly attach to Gram-negative bacteria *E. coli* and *Klebsiella pneumoniae*; Gram-positive bacteria *Bacillus subtilis*, *Bacillus thuringiensis* and *Staphylococcus aureus*; fungi *Candida albicans* and *Saccharomyce cerevisiae*, and only domain 1 was responsible for the binding to *E. coli* and *S. aureus*. In addition, recombinant hcPcSPI1 was also found to possess bacteriostatic activity against the *B. subtilis* and *B. thuringiensis*. Domains 2 and 3 contributed mainly to these bacteriostatic activities. All results suggested that hcPcSPI1 might play important roles in the innate immunity of crayfish.

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1. Introduction

Serine proteinases inhibitors (SPIs) are widely found in organisms. They are involved in various biological processes including prophenoloxidase activation, blood coagulation, complement system, metamorphosis and development, among others [1–5]. Recent report has categorized SPIs into at least 59 families [6]. Four SPI family members, Kazal, Kunitz, Serpin and α-macroglobulin, are believed to participate in the immune responses [1,7,8]. In the immune responses, SPIs have been documented to serve as defense components by inhibiting the proteinases excreted from pathogenic fungi and bacteria [9,10], or by functioning as regulators of the host-defense reaction by deactivating the excessive proteinase activities from blood clotting and the complement system [11].

Kazal-type inhibitors with one or more Kazal domains have been characterized by a well-conserved motif in their amino acid sequences [12] and highly homologous three-dimensional structures [13]. Each Kazal domain usually contains six conserved cysteine residues forming three disulfide bonds [12,14]. P1 residue, which contributes mainly to the inhibitory specificity, is located at the second position after the second cysteine residue of the Kazal domain [15]. Many studies on inhibition mechanism have proven that most Kazal-type inhibitors react with cognate enzymes according to a substrate-like standard mechanism [12].

To date, over 200 Kazal-type inhibitors have been identified in vertebrates, invertebrates, and bacteria [6]. Many of these have been demonstrated to have a variety of functions *in vivo* [16,17]. In invertebrate animals, the twelve-domain SPI from the Zhikong scallop [18] and the three-domain SPI from Hydra [19] have recently been reported. Several Kazal-type inhibitors have also been reported in crustaceans [20–25]. The functions of some of these SPIs were investigated through recombinant or purified proteins *in vitro*. For instance, it has been found that four-domain SPI from freshwater crayfish could inhibit chymotrypsin and subtilisin [20]; five-domain SPI from the black tiger shrimp could inhibit subtilisin, elastase, and trypsin, as well as the growth of *B. subtilis* [26]; and three-domain SPI from Hydra could inhibit

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subtilisin and trypsin, and could kill *S. aureus* directly [19]. All these inhibitors have been proven to be involved in the innate immune response of invertebrates.

Freshwater crayfish is normally used as a model to study the innate immune system of invertebrate [27–29]. In our laboratory, a cDNA library was constructed from the hepatopancreas and gills of the crayfish, through which we found that there were several Kazal-type serine proteinase inhibitors in the crayfish. In the present study, a Kazal-type serine proteinase inhibitor named hcPcSPI1 was chosen for the detailed analysis. The tissue distribution and expression profiles of hcPcSPI1 were investigated. Subsequently, this inhibitor and each domain were overexpressed to study the biochemical characteristics, microorganism-binding activity, and bacteriostatic activity. The results showed that hcPcSPI1 might play important roles in the innate immunity of crayfish, especially by defending the bacterial pathogens.

2. Materials and methods

2.1. Challenge of crayfish and collection of tissues

The freshwater crayfish, *Procambarus clarkii*, were taken from a market in Jinan, Shandong Province, China and were temporarily kept in tanks in air-pumped water at 16 °C. For immune stimulations, 20 μl of *E. coli* (8099) suspension (1×10^7 colony forming units) or 50 μl of WSSV inocula (1×10^5 copies) were injected into the abdominal segment of each crayfish, and the control was challenged with PBS or the supernatant of normal tissues. From the ventral sinus, the hemolymph was collected at a required time using ice-cold anticoagulant buffer (0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA, pH 4.6) [30]. It was centrifuged immediately at $800 \times g$ for 10 min (4 °C) to isolate the hemocytes. The cell-free hemolymph was obtained simultaneously. Subsequent to the collection of hemolymph, the crayfish tissues were obtained through dissection and immediately stored at −80 °C.

2.2. cDNA cloning

A cDNA library was constructed using hepatopancreas and gills of crayfish challenged by WSSV. Following the manufacturer's instruction, the total RNA was extracted from the hepatopancreas and gills of 96 h infected crayfish using the Unizol reagent (Biostar, Shanghai, China). Messenger RNA (mRNA) was extracted by PolyA Tract mRNA isolation system (Promega, USA) and then used to construct the cDNA library. The Creator SMART cDNA Library Construction Kit (Clontech, USA) was used for the cDNA library construction following the manufacturer's instruction. The double strand cDNA was ligated with the pDNR-LIB vector, and then transformed into the competent cells DH5α. The individual colonies were randomly selected, and plasmid was extracted for sequencing. Through this process, the full length of hcPcSPI1 was obtained.

2.3. Sequence analysis

The similarity of hcPcSPI1 and other Kazal-type SPIs was analyzed using the online BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi/). Gene translation and deduced protein forecast were conducted using ExPASy (http://www.expasy.ch/). Signal peptide searching and motif forecasting were carried out using SignalP [31] and SMART (http://smart.embl-heidelberg.de/), respectively. Furthermore, multiple alignment of Kazal-type domains and phylogenetic tree were performed with the MEGA 4 [32] and GENEDOC software. The active sites were predicted via homolog sequence alignment. The *cell attachment sequence* was predicted by ExPASy PROSITE (http://www.expasy.org/prosite/).

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

Total RNA was isolated using Unizol (Biostar, Shanghai, China) from different tissues of normal crayfish, and from the hemocytes at 0, 2, 6, 12, and 24 h after bacterial injection or 0, 6, 12, 24, 48, 72, and 96 h after viral injection. Next, the total RNA (5 μg) was reverse transcribed and the synthesized first strand cDNAs were used as the templates for semi-quantitative RT-PCR and quantitative real-time PCR (qRT-PCR). Tissue distribution was studied using semi-quantitative RT-PCR analysis. Primers F1 and R1 were applied to generate the target fragment of hcPcSPI1. The primers SF and SR were used to amplify 18sRNA as the reference (Table 1). The PCR procedure was 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 finally 72 °C for 10 min.

The qRT-PCR was performed to detect the temporal expression of hcPcSPI1 after bacterial or viral challenge following the methods described in a previous report [33]. Two pairs of primers (F1, R1) and (SF1, SR1) were used to amplify hcPcSPI1 and the control 18sRNA, respectively. The qRT-PCR was programmed at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 30 s, 72 °C for 20 s, and then melting from 60 to 95 °C. All tests were repeated three times using individual templates. The obtained data were subjected to statistical analysis followed by an unpaired sample *t*-test. A significant difference was accepted at $P < 0.05$.

2.5. Recombinant expression, purification, and antiserum preparation of hcPcSPI1

Expression vectors of hcPcSPI1 and each domain (Domain 1, 2, and 3) were constructed. The DNA fragments, corresponding to amino acid residues 18–168 for hcPcSPI1, 18–70 for Domain 1, 72–121 for Domain 2, and 122–168 for Domain 3 were ligated into pET 30a(+)vector (Novagen). The primers (EF, ER; EF, D1R; D2F, D2R; and D3F, ER) for amplification of the four fragments are listed in Table 1. The expression vector of hcPcSPI1 was transformed into competent *E. coli* BL21 (DE3) cells, while the others were transformed into *E. coli* Rosetta (DE3) host cells. IPTG was added with a final concentration of 0.5 mM to induce protein expression at 28 °C for 10 h. After induction, the bacteria were collected by centrifugation for 8 min at 6000 rpm. The bacterial pellets were

Table 1
Sequences of the primers used in this study.

Primers	Sequence (5′-3′)
hcPcSPI1	
F1	CATTCGCCTTAATCGCCTTCT
R1	GACATTTCCCTTCATAATCCACC
EF	TACTCAGAATTCCAACGGCATGAGTGCATCA
ER	TACTCACTCGAGGGACTCTTAACATTCACCCT
D1R	TACTCACTCGAGTTAGTCACCACGACATTTCCCT
D2F	TACTCAGAATTCGAATGCCCGTCAGCCTGCATCT
D2R	TACTCACTCGAGTTAGTTGTCACCACATTGACCATT
D3F	TACTCAGAATTCGAATGCCCGATAGCTTGTACTG
18sRNA	
SF	TGGTGCATGGCCGTTCTTA
SR	AATTGCTGGAGATCCGTCGAC
SF1	ACCGATTGAATGATTTAGTGAG
SR1	TACGGAAACCTTGTTACGAC
Universal primers	
M13F	TGTAAAACGACGGCCAGT
M13R	AAACAGCTATGACCATGTTCA

The underlined nucleotides indicate the locations of restricted endonucleases (EcoR I or Xho I).

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