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Identification and characterization of a serine protease inhibitor Esserpin from the Chinese mitten crab *Eriocheir sinensis*

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A B S T R A C T

Serine protease inhibitors (serpins) represent an expanding superfamily of endogenous inhibitors that regulate proteolytic events and involve in a variety of physiological processes. A serine protease inhibitor, namely Esserpin, was identified from Chinese mitten crab *Eriocheir sinensis* based on expressed sequence tag (EST) analysis. The full-length cDNA of Esserpin was of 2367 bp, including an open reading frame (ORF) of 1371 bp encoding a polypeptide of 456 amino acids with estimated molecular mass of 49.95 kDa and theoretical isoelectric point of 6.03. A putative signal peptide of 23 amino acids and a classical serpin domain were identified in Esserpin. The deduced amino acid sequence of Esserpin shared homology with serpins from *Fenneropenaeus chinensis* and *Pacifastacus leniusculus*. The mRNA transcripts of Esserpin could be detected in all the examined tissues including heart, gill, hemocytes, muscle, gonad and hepatopancreas, and the highest expression level was present in gonad. After the crabs were challenged by *Vibrio anguillarum* and *Pichia pastoris*, the expression levels of Esserpin transcripts in hemocytes were significantly up-regulated, and peaked at 24 h (5.18-fold of blank group, $P < 0.05$) and 3 h (2.87-fold of blank group, $P < 0.05$), respectively. The functional activity of Esserpin was investigated by recombination and expression of the cDNA fragment encoding its mature peptide in *Escherichia coli* BL21 (DE3)-pLysS. The recombinant Esserpin (rEsserpin) could inhibit trypsin activities in a dose-dependent manner, and it could lead to 100% inhibition of trypsin activities under the concentration of 873.76 nM, while there was no evident inhibition of chymotrypsin observed with rEsserpin. Moreover, rEsserpin inhibited the growth of *E. coli* at the final concentration of 1747.52 nM, and it also significantly depressed ($P < 0.05$) the phenoloxidase activity in the plasma at the final concentration of 873.76 nM. These results indicated that Esserpin was a homologue of serpin in crab and it could be induced after immune stimulation and mediate immune response possibly via the inhibition of bacterial growth and the regulation of prophenoloxidase-activating system.

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1. Introduction

The innate immune system is considered to constitute an evolutionarily older defense strategy, which comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner [1]. Many well-known innate immune processes including blood coagulation, complement activation, melanization, phagocytosis, encapsulation, and synthesis of antimicrobial peptides are mediated by the protease cascades leading to multiple steps of protease activation [2–4]. Serine protease inhibitors (SPIs) are group of protease inhibitors, which play important roles as modulators of several immune processes by inactivating the excessive protease activities [5–13].

Serpins, as important members of SPI superfamily [14], are of interest due to a diverse set of functions including, but not limited to, inhibition of serine proteases in the vertebrate blood coagulation cascade [15,16] and the invertebrate prophenoloxidase cascade [4]. Several hundreds of serpins have been identified so far in higher eukaryotes, bacteria and viruses. They act as suicide-like substrates to irreversibly inhibit the specific target proteases and actively involve in numerous important biological processes, such as blood coagulation, fibrinolysis and inflammation [14]. Serpins have a relatively large molecular weight of 40–60 kDa, and they all contain a conserved protein spatial structure folded by three β-sheets and seven to nine α-helices with a reactive centre loop

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(RCL) near their C-terminus [17]. The RCL, an exposed protein motif, contains a scissile bond between two residues, called P1 and P1′, which can be cleaved by the target protease. The P1 residue is the key amino acid residue that determines the target specificity. The RCL of serpin can be inserted into the active site of a specific target protease to form a complex and lead to a large conformational change. Finally, the interaction between the serpin and protease results in the inactivation of the protease activity [18,19].

Recently, serpins have been identified and characterized in some arthropod, which have been found mainly involved in the response against infection and regulation of prophenoloxidase-activating system (proPO system) [20–30]. For instance, the mRNA transcripts of PmSERPIN8 from *Penaeus monodon* [27] and PtSerpin from *Portunus trituberculatus* [28] could be up-regulated or altered in response to pathogenic infection, and PmSERPIN8 also displayed the capability to inhibit bacterial growth. In *Drosophila melanogaster*, serpin-28D and serpin-27A were reported to control the activation of proPO system, and the deficiency in serpin-28D led to high melanin production [24,30]. Several serpins from *Manduca sexta* were also found to be involved in the regulation of prophenoloxidase cascade [22,23]. Although invertebrate serpins have been proposed to play important roles in many biological processes, the information about function of crustacean serpins in the immune response is still very limited.

Chinese mitten crab *Eriocheir sinensis* (Henri Milne Edwards 1854) is one of the most important cultivated species in Southeast Asia [31]. In the past years, the frequent outbreak of diseases has led to drastic decreases of production and catastrophic economic losses. Accumulating evidences demonstrate that the cognition of protease inhibitors can shed new light on the interaction of pathogens with their host [3]. Study of the protease inhibitors such as serpins in crabs may be conducive to the knowledge of immune defense mechanisms as well as the development of better disease management strategies in crustacean farming. The main objectives of the present study were (1) to clone the full-length cDNA of serpin from crab *E. sinensis* (designated as Esserpin), (2) to investigate its expression in various tissues and its temporal response to pathogenic infection at mRNA level, (3) to test its inhibitory activity on different proteases and (4) to examine its possible function in the inhibition of bacterial growth and the regulation of prophenoloxidase-activating system.

2. Materials and methods

2.1. EST analysis and cloning of the full-length cDNA of Esserpin

A cDNA library was constructed from the hemocytes of *E. sinensis* challenged with *Vibrio anguillarum* and *Staphylococcus aureus*, using the ZAP-cDNA synthesis kit and ZAP-cDNA GigapackIII Gold cloning kit (Stratagene, USA). Random sequencing of the library using T3 primer (Table 1) yielded 7535 successful sequencing reactions [32]. BLAST analysis revealed that one EST (Contig55) was homologous to serpin from *Pacifastacus leniusculus* (CAA57964.1), and it was selected for further study. The clone corresponding to the EST mentioned above was completely sequenced by using T7 primer (Table 1), and the cDNA sequences generated from the corresponding clone and Contig55 were assembled and analyzed.

2.2. Sequence analysis

Nucleotide and protein sequence similarities were searched with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). SignalP 3.0 was utilized to predict the signal peptide (http://www.cbs.dtu.dk/services/SignalP/). The protein motif features were predicted by Simple Modular Architecture Research Tool (http://smart.emblheidelberg.de/). Multiple alignment of the Esserpin was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). A phylogenic NJ tree was constructed with Mega 4 program [28,33]. To derive the confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

Table 1
Primers used in this study.

Primer	Sequence (5′-3′)	Sequence information
P1 (forward)	TCTACGGGTCCTGGGAAACA	Real-time Esserpin primer
P2 (reverse)	TGTAGGGCAGACGCAGAACTC	Real-time Esserpin primer
P3 (forward)	GCATCCACGAGACCACTTACA	Real-time β-actin primer
P4 (reverse)	CTCCTGCTTGCTGATCCACATC	Real-time β-actin primer
P5 (forward)	GGTACCATCGTGGATGACAT AACAGGGGCTA	Recombinant primer
P6 (reverse)	GAATTCTTAGGCCTTCGGGGG GGTCTTG	Recombinant primer
T3	AATTAACCCTCACTAAAGGG	Vector primer
T7	GTAATACGACTCACTATAGGGC	Vector primer
Oligo(dT)-adaptor	GGCCACGCGTCGACTAGTACT$_{17}$	Clone primer

2.3. Crabs, microorganism challenge and hemocytes collection

Healthy *E. sinensis* averaging 50 g in weight were collected from a farm in Qingdao, China, and cultured at 22–27 °C for 1 week prior to experimentation. For the bacterial and fungal challenge experiment, 120 crabs were employed and kept in aerated fresh water, and they were randomly divided into 3 groups with 40 individuals in each group. The crabs in two groups were immersed in *V. anguillarum* (Bacteria) or *Pichia pastoris* (Fungus) (1×10^7 CFU mL^{-1})-contained freshwater, and the crabs in the fresh water without any treatment were employed as the blank group. Six crabs were randomly sampled from each group at 1.5, 3, 6, 12, 24 and 48 h post challenge. The hemolymph (about 1.0 ml per individual) was harvested from cheliped using a syringe with an equal volume of anticoagulant (27 mM sodium citrate, 336 mM NaCl, 115 mM glucose, 9 mM EDTA, pH 7.0) [34], and centrifuged at 800 g, 4 °C for 10 min to collect the hemocytes for RNA extraction. The heart, gill, hemocytes, hepatopancreas, muscle, and gonad from seven untreated crabs were collected to determine the tissue distribution of Esserpin transcripts. Total RNA was immediately extracted using Trizol reagent according to the manufacture's protocol (Invitrogen, USA).

2.4. Real-time PCR analysis of Esserpin mRNA expression

The temporal expression of Esserpin in hemocytes of crabs challenged with *V. anguillarum* or *P. pastoris* and the mRNA expression of Esserpin in various tissues were determined by quantitative real-time RT-PCR. Total RNA was extracted according to the protocol of Trizol (Invitrogen, USA) and quantified by measuring the absorbance at 260 nm [35]. The synthesis of first-strand cDNA was carried out based on the Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo (dT)-adaptor primer (Table 1). The cDNA mix was incubated at 42 °C for 1 h, terminated by heating at 95 °C for 5 min, diluted to 1:100 by the DEPC-treated water, and then stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

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