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Kazal-type serine proteinase inhibitors from the black tiger shrimp *Penaeus monodon* and the inhibitory activities of SPI*Pm*4 and 5

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ABSTRACT

Serine proteinase inhibitors (SPIs) play important roles in physiological and immunological processes involving proteinases in all multicellular organisms. In black tiger shrimp *Penaeus monodon*, nine different Kazal-type SPIs, namely SPI*Pm*1-9, were identified from the cDNA libraries of hemocyte, hepatopancreas, hematopoietic tissue, ovary and lymphoid organ. They are multi-domain SPIs containing 2–7 and possibly more Kazal domains. Two interesting cDNA clones, SPI*Pm*4 and SPI*Pm*5 coding for two-domain Kazal-type SPIs, were identified from the heat-treated hemocyte cDNA libraries. The SPI*Pm*4 and SPI*Pm*5 consist of open reading frames of 387 and 399 bp coding for polypeptides of 128 and 132 amino acids with putative signal peptides of 21 and 19 amino acid residues and mature SPIs of 107 and 113 amino acid residues, respectively. Recombinant expression in an *Escherichia coli* expression system yielded recombinant proteins, rSPI*Pm*4 and rSPI*Pm*5, with molecular masses of 12.862 and 13.433 kDa, respectively. The inhibitory activities of SPI*Pm*4 and SPI*Pm*5 were tested against trypsin, chymotrypsin, subtilisin and elastase. The SPI*Pm*4 exhibited potent inhibitory activity against subtilisin and weakly against chymotrypsin whereas the SPI*Pm*5 strongly inhibited subtilisin and elastase. The inhibition was a competitive type with inhibition constants (K_i) of 14.95 nM for SPI*Pm*4 against subtilisin, 4.19 and 59.64 nM, respectively, for SPI*Pm*5 against subtilisin and elastase. They had no bacteriostatic effect against Gram-positive bacteria: *Bacillus subtilis*, *Bacillus megaterium*, *Staphylococcus aureus*, and Gram-negative bacteria: *Vibrio harveyi* 639, *E. coli* JM109. Gene expression study revealed that the SPI*Pm*5 gene was up-regulated in response to heat treatment suggesting the involvement of SPIs in stress responses.
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1. Introduction

In multicellular organisms, serine proteinase inhibitors (SPIs) are essential factors involving in controlling the various proteinase-mediated biological processes, such as the complement system, blood coagulation, melanization, apoptosis, etc. [1,2]. Not only do they control the extent of deleterious protease digestion in such processes, they potentially fight as part of the humoral defence of the innate immune system against the invading pathogens [3]. For example, a subtilisin inhibitor, BmSPI, from *Bombyx mori* might function as an inhibitor to the microbial proteases and protected the silkworm pupae from infection by pathogens [4].

Some microbial pathogens and parasites use the SPIs to counter-defense the host protective proteinases. For example, the oomycete *Phytophthora infestans*, a cause of disease in potato and tomato, produces an extracellular protease inhibitor to counter-defence the plant defensive proteinases [5,6]. The obligate intracellular parasite of human *Toxoplasma gondii* produces a serine protease inhibitor to protect itself from the digestive enzymes during its residency in small intestine [7]. Some other SPIs are involved in reproductive processes. A male reproduction-related SPI is isolated from *Macrobrachium rosenbergii* with inhibitory activity on sperm gelatinolytic activity [8]. Another reproductive SPI was from the turkey male reproductive tract [9]. For hematophagous insects such as *Dipetalogaster maximus* and *Triatoma infestans*, they secrete potent thrombin inhibitors dipetalogastin and infestin, respectively, to prevent blood clotting during blood meal [10,11].

Since SPIs are widely distributed and more are to be found and characterized, to facilitate the studies in this field, they were classified on the basis of amino acid sequence similarities into at least 63 families (http://merops.sanger.ac.uk/) [12,13]. One of the well known SPIs is the Kazal-type SPIs which are grouped into family I1. The Kazal inhibitors are usually multi-domain proteins containing more than one Kazal domain. Each domain of 50–60 amino acid

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residues contains six well-conserved cysteine residues capable of forming three intra-domain disulphide bridges resulting in a characteristic three-dimensional structure [14]. Each domain binds tightly and competitively via its reactive site loop to the active site of cognate proteinase rendering the proteinase inactive. Structural studies reveal that there are several contact positions responsible for the interactions between Kazal domains and the proteinases [15,16]. However, the inhibitory specificity is determined mainly by the P1 amino acid residue resided at the second amino acid residue after the second cysteine residue of the domain.

Recently, two Kazal SPIs are identified from scallop and shrimp. They were believed to be involved in host defence mechanism for they were up-regulated upon microbial infection. The novel 12-domain Kazal-type SPI from the Zhikong scallop *Chlamys farreri* SPI was isolated. Its expression in hemocytes was up-regulated upon *Vibrio anguillarum* challenge [17]. The two-domain Kazal-type SPI from the oriental white shrimp *Fenneropenaeus chinensis* was characterized. Its expression was up-regulated in the white spot syndrome virus (WSSV) infected shrimp [18].

For the black tiger shrimp *Penaeus monodon*, a few SPIs had been reported before the *P. monodon* EST database was established, particularly the SPI*Pm*2 [19,20]. The EST approach was later used to identify more genes from the shrimp. A total of 40,001 EST clones were collected and sequenced leading to the establishment of the EST database (http://pmonodon.biotec.or.th/home.jsp) [21]. Upon clustering of the EST clones to remove the redundancy, 10,536 unique genes were obtained. In this study, we searched the cDNA clustering databases for the Kazal-type SPIs. Analyses of the nucleotide sequences of contigs and their EST clone members enabled us to classify the Kazal inhibitors in the black tiger shrimp. Two interesting SPIs, SPI*Pm*4 and 5, were over-expressed and their inhibitory activities towards proteinases studied. The expression of SPI*Pm*5 gene in response to heat stress was also tested.

2. Materials and methods

2.1. Searching the P. monodon EST database

The contig pages in the *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) was searched for the Kazal-type serine proteinase inhibitors. The nucleotide sequences of the obtained contigs and singletons were analyzed for the open reading frames and the encoded amino acid sequences. The EST clones containing the entire open reading frames of the interesting contigs were re-sequenced to ensure the correctness of the sequence data. SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal cleavage sites [22]. ClustalX was used to align the sequences from different contigs and EST clones [23].

2.2. Expression analysis of SPIPm5 using RT-PCR

Three-month-old subadult black tiger shrimp, *P. monodon*, of about 15–20 g weight, were obtained from a local farm and acclimatized in aquaria at an ambient temperature of about 28 ± 1 °C and a salinity of 15 ppt for a few days before the experiments. The shrimp were divided into two groups, control and heat-treated groups. Each group consisted of 3 subgroups of 3 individuals. The heat-treated group was immediately put and reared for 1 h in an aquarium with warm water of 33 ± 1 °C. Then, the hemolymph was collected from the shrimp ventral sinus into an anticoagulant solution of 10% (w/v) trisodium citrate dihydrate, pH 4.6. Hemocytes were pelleted by centrifugation, resuspended in TRI REAGENT® (Molecular Research Center), homogenized and total RNA isolated. The equivalent amounts of total RNA preparations from individuals in each subgroup were pooled and treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. The concentration and integrity of total RNA were assessed by UV spectrophotometry and agarose gel electrophoresis. First-strand cDNAs were synthesized from 1 μg of total RNA samples using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol.

RT-PCR analysis was carried out using the SPI*Pm*5 specific primers: FSPI*Pm*5, 5′ TGGAACGGACGGCAAGACAT 3′ and RSPI*Pm*5, 5′ GTCGTGACAGTCTTGAGTCC 3′. The β-actin gene was used as an internal control using the gene specific primers designed according to the shrimp actin cDNA sequence (GenBank accession no. DW042525): actinF, 5′ GCTTGCTGATCCACATCTGCT 3′ and actinR, 5′ ATCACCATCGGCAACGAGA 3′. Each PCR reactions were carried out in a total volume of 25 μl containing 10 mM Tris–HCl, pH 8.8, 50 mM $(NH_4)_2SO_4$, 0.1% (v/v) Triton X-100, 2 mM $MgCl_2$ for SPI*Pm*5 or 1 mM for β-actin, 0.2 mM of each dNTP, 0.4 μM of each primer for SPI*Pm*5 or 0.2 μM of each primer for β-actin, 1.5 unit of DyNAzyme™ II DNA Polymerase (Finnzymes) and 3 μl of undiluted template cDNA for SPI*Pm*5 or 3 μl of 10-fold diluted template cDNA for β-actin. The reactions were predenatured at 94 °C for 2 min followed by 40 cycles for SPI*Pm*5 or 25 cycles for β-actin of denaturation at 94 °C for 30 s, annealing at 60 °C for SPI*Pm*5 or 52 °C for β-actin for 30 s and extension at 72 °C for 1 min. The final extension was at 72 °C for 5 min. The PCR products were analyzed using a TBE-2% agarose gel electrophoresis.

2.3. Construction of the protein expression clones

To express the recombinant SPI, two primer pairs, FPm4: 5′ AA GGCCATGGGAAAGGGGGGGGATTCTCGACT 3′ and RPm4: 5′ CCTT CTCGAGATATCCCGTCTTCCTGTCAA 3′ for SPI*Pm*4; FPm5: 5′ AAGG CCATGGGAAAAGGAGGCAAATTCAGACT 3′ and RPm5: 5′ CCTTCTCG AGATATCCCTTCTTGATAGGCG 3′ for SPI*Pm*5, were designed. The primers were designed for the amplification of gene fragment encoding the mature proteins without signal peptides (Fig. 1). The included NcoI and XhoI sites (underlined), respectively, at the 5′ and 3′ ends of the gene fragments were for cloning into the expression vector. For convenience, the EST clones from the normalized hemocyte library, HC-N-N01-4898-LF (GenBank accession no. GO269555) and HC-N-N01-2619-LF (GenBank accession no. GO269556) containing the complete open reading frames of SPI*Pm*4 and 5, respectively, were used as PCR templates.

The SPI*Pm*4 and 5 gene fragments were PCR amplified in a final reaction volume of 50 μl containing 0.02 ng of plasmid template, 0.4 M of each primer, 0.2 mM of each dNTP and 3 units of *Pfu* polymerase (Promega). The PCR amplification was carried out at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and final extension at 72 °C for 7 min. The amplified products were analyzed using 1.2% agarose gel electrophoresis, excised and purified using NucleoSpin® Extract II Kits (Macherey-Nagel). The purified DNA fragments were tailed with an adenine nucleotide for ligation into the T&A cloning vector (Real Biotech Corporation). The resulting T&A clones were isolated and subjected to nucleotide sequencing to confirm the insert sequences of SPI*Pm*4 and 5.

The NcoI–XhoI fragments containing the SPI*Pm*4 and 5 were prepared from the T&A clones and subcloned into the NcoI–XhoI digested pET-28b(+) expression vector (Novagen). For some reasons, the SPI*Pm*4 was not expressed well; it was then subcloned into a pET-32a(+) derivative, pVR500 [24], and expressed as fusion protein to the thioredoxin. The expression clones were named pSPI*Pm*4 and 5.

2.4. Preparation of the recombinant proteins

The expression clones, pSPI*Pm*4 and 5, were transformed into an *Escherichia coli* Rosetta(DE3)pLysS. A single colony was cultured in

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