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A Kazal-type serine proteinase inhibitor SPI*Pm2* from the black tiger shrimp *Penaeus monodon* is involved in antiviral responses

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ABSTRACT

A five-domain Kazal-type serine proteinase inhibitor, SPI*Pm2*, from *Penaeus monodon* has recently been implicated in antiviral responses for it is up-regulated upon viral infection and needs further studies. The *SPIPm2* genomic gene was composed of seven exons and six introns. The genomic DNA segments coding for each Kazal domain were separated by introns of variable lengths supporting the hypothesis of gene duplication in the Kazal-type gene family. RT-PCR and Western blot analysis revealed that the *SPIPm2* transcript and its five-domain protein product were expressed mainly in the hemocytes and less in gill, heart and antennal gland. Upon white spot syndrome virus (WSSV) infection, the SPI*Pm2* was only detected in the hemocytes and plasma. Immunocytochemical study of *P. monodon* hemocytes showed that the percentage of SPI*Pm2*-producing hemocytes was reduced by about half after WSSV infection. Quantitative RT-PCR revealed further that the SPI*Pm2* was up-regulated early in the hemocytes of WSSV-infected shrimp and gradually reduced as the infection progressed. Injection of the recombinant SPI*Pm2* (rSPI*Pm2*) prior to WSSV injection resulted in a significant inhibition of WSSV replication. The rSPI*Pm2* injection also prolonged the mortality rate of WSSV-infected shrimp. Therefore, the SPI*Pm2* was involved in the innate immunity against WSSV infection in shrimp.

1. Introduction

All multicellular organisms possess innate immunity as the first line of defense against the invading and potentially harmful non-self entities. The innate immune responses include phagocytosis, complement fixation, blood coagulation, melanization and synthesis of immune-active peptides and proteins (Cerenius and Söderhäll, 2004; Jiravanichpaisal et al., 2006). Some of the aforementioned innate immune processes are modulated by ubiquitous proteinases and proteinase inhibitors as parts of the homeostasis in organisms (Kanost, 1999; Rimphanitchayakit and Tassanakajon, 2010). The proteinase inhibitors function to limit and control the extent of proteinase activities in the processes. Proteinases and proteinase inhibitors also function in many other biological processes, such as metamorphosis, food digestion, in the organisms (Laskowski and Kato, 1980; Roberts et al., 1995).

One of the well-known inhibitor families is the family I1 (http://merops.sanger.ac.uk/) (Rawlings et al., 2008), which is a family of the Kazal-serine proteinase inhibitors (KPIs). The KPIs typically contain one or more Kazal domains capable of binding to the proteinases (Rimphanitchayakit and Tassanakajon, 2010). The 'canonical' inhibition commences by tight binding of the KPI reactive site loop into the active site of cognate proteinase, competitively blocking and rendering the enzyme inactive (van de Locht et al., 1995).

Previously, the KPIs were implicated in host defenses against certain pathogens. The two KPIs from *Galleria mellonella* and *Fenneropenaeus chinensis* were able to inhibit the bacterial subtilisin and fungal proteinase K (Nirmala et al., 2001a,b; Wang et al., 2009). Besides the proteinase inhibitory activity, some KPIs possessed antimicrobial activity such as those isolated from *Procambarus clarkii* and *Hydra magnipapillata* (Augustin et al., 2009; Li et al., 2009). Some KPI genes were up-regulated upon bacterial or viral challenges. For instances, KPI genes from *Argopecten irradians* and *Chlamys farreri* were up-regulated following *Vibrio anguillarum* injection (Wang et al., 2008; Zhu et al., 2006). A two-domain KPI gene from *F. chinensis* was up-regulated after challenging with the white spot syndrome virus (WSSV) (Kong et al., 2009).

In *Penaeus monodon*, there are at least 9 types of KPIs identified from the EST database (Tassanakajon et al., 2006; Visetnan et al., 2009). A five-domain KPI, SPI*Pm2*, is the most abundant KPI and implicated in host defense against pathogens (Visetnan et al., 2009; Supungul et al., 2002). It possesses strong inhibitory activities against subtilisin and elastase, and weakly against trypsin

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Table 1
Primer pairs used to amplify the SPI*Pm*2 genomic sequences and for RT-PCR.

Primer	Sequence (5′–3′)	Position	PCR fragment
SPIPm2_Signal	GATGGCCAACAAAGTGGCAC	Start codon	Start codon to domain 2
SPIPm2_D2R	GCACCGTTGCAGGTTCTGTC	Domain 2	
SPIPm2_D2F	CGAACTTATGCCAACTTGAG	Domain 2	Domain 2 to domain 3
SPIPm2_D3R	GCTGCCTGGAAATAGCAGTC	Domain 3	
SPIPm2_D3F	TGTGGCAGTGATGGCAAGAC	Domain 3	Domain 3 to stop codon
SPIPm2_Stop	CCACATCTCTCTTTAATATC	Stop codon	
FPm2	ATGCAACCACGTCTGTACTG	–	RT-PCR
RPm2	CTGCAAGGTTCCACATCT	–	
actinF	GCTTGCTGATCCACATCTGCT	–	Internal control for RT-RCR
actinR	ATCACCATCGGCAACGAGA	–	

(Somprasong et al., 2006). While the domain 1 is inactive, other domains are responsible for the proteinase inhibitory activities. The SPI*Pm*2 also possesses bacteriostatic activity against *Bacillus subtilis* (Donpudsa et al., 2009).

Recently, the *SPIPm*2 was found to be up-regulated upon yellow head virus (YHV) and WSSV infection. Using the suppression subtractive hybridization approach, several immune-related genes including the *SPIPm*2 were up-regulated after YHV infection (Prapavorarat et al., 2010). The cDNA microarray experiment, which was carried out to compare the expression profiles between infected and un-infected shrimp, revealed that the up-regulation of *SPIPm2* was induced by YHV and WSSV but not *V. harveyi* (unpublished). The SPI*Pm*2 might be, in some ways, involved in the defense against the viral pathogens. To determine its potential immune defense against the major shrimp pathogen WSSV, we, therefore, experimented on the SPI*Pm*2 for its expression upon infections and its affect on WSSV replication. Its genome organization was also determined.

2. Materials and methods

2.1. Genome organization

Genomic DNA was prepared from the pleopods of *P. monodon* using a standard phenol–chloroform extraction. The sequence of the *SPIPm*2 gene was determined by PCR amplification technique using genomic DNA as a template and six primers (Table 1), designed from the cDNA of *SPIPm*2 (GenBank accession BI018075), to amplify DNA from the start codon to the second domain, the second domain to the third domain and the third domain to the stop codon of SPI*Pm*2.

Approximately 50–100 ng of template DNA was used for the PCR amplification in 50 μl reaction containing one unit Advantage 2 Polymerase Mix (Clonetech), 1× Advantage 2 buffer, 0.4 mM each dNTP, 0.2 mM each primer. The reaction started with an initial denaturation step at 94 °C for 2 min, followed by: 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 68 °C for 3 min. The expected DNA fragment was cloned into the T&A vector (RBC) and sequenced in both directions by a commercial service Macrogen Inc., Korea. The sequences of PCR products were compared with that of *SPIPm*2 cDNA in order to determine the introns, exons and 5′/3′ splice sites.

2.2. Preparation of rSPIPm2

An *E. coli* Rosetta(DE3)pLysS transformant containing the rSPI*Pm*2 expression plasmid, pSPIPm2-NS2 (Somprasong et al., 2006), was cultured under vigorous shaking at 37 °C until the optical density at 600 nm reached 0.6–0.8. The protein expression was induced by adding IPTG to the final concentration of 1 mM and the incubation was continued for 4 h. The cell pellet was collected by centrifugation at 8000 × *g* for 10 min, frozen completely at −80 °C and thawed at room temperature for three times and resuspended in a lysis buffer (50 mM Tris–HCl pH 8, 5% glycerol and 50 mM NaCl). The suspension was sonicated with a Bransonic 32 (Bandelin) for 4 min and centrifuged at 10,000 rpm for 20 min to remove the supernatant liquid. The pellet containing the inclusion bodies was washed twice with 0.5 M NaCl, 2% Triton X-100, twice with 0.5 M NaCl and twice with distilled water. The inclusion bodies were solubilized with 50 mM sodium carbonate buffer pH 10 at room temperature overnight. The insoluble material was removed by centrifugation. The soluble protein was purified using a Ni-NTA agarose column. The purified rSPI*Pm*2 was dialyzed against 50 mM sodium carbonate buffer pH 10 or distilled water. The protein was analyzed using SDS-PAGE. The concentration of protein was determined using the Bradford method (Bradford, 1976).

2.3. Rabbit serum and anti-SPIPm2 immune serum

Rabbit serum was purchased from the National Laboratory Animal Centre, Mahidol University, Thailand. Rabbit polyclonal antiserum was raised against rSPI*Pm*2 using 2 mg of the purified recombinant protein. It was performed in the Biomedical Technology Research Unit, Faculty of Associated Medical Sciences, Chiang Mai University, Thailand.

2.4. Expression of SPIPm2 transcript and protein in different tissues

The expression of SPI*Pm*2 transcript in ten shrimp tissues was determined by RT-PCR analysis using the *SPIPm*2 specific primers: FPm2 and RPm2 (Table 1). The shrimp tissues: intestine, heart, hepatopancreas, hemocyte, gill, eyestalk, epipodite, antennal gland, lymphoid and stomach, were collected, homogenized in TRI Reagent® (Molecular Research Center), and total RNA isolated. The total RNA preparations were treated with RNase-free DNase I (Promega) to remove contaminated genomic DNA. 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. The shrimp *β-actin* gene was used as an internal control using the gene specific primers: actinF and actinR (Table 1).

Each PCR reaction was 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, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer for *SPIPm*2 or 0.2 μM of each primer for *β-actin*, 1.5 unit of DyNAzyme™ II DNA Polymerase (Finnzymes) and 1 μl of undiluted template cDNA for *SPIPm*2 or 3 μl of tenfold diluted template cDNA for *β-actin*. The reactions were denatured at 94 °C for 2 min followed by 32 cycles for *SPIPm*2 or 25 cycles for *β-actin* of denaturation at 94 °C for 30 s, annealing at 60 °C for *SPIPm2* 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 agarose gel electrophoresis. The PCR products were 192 and 326 bp DNA fragments for *SPIPm*2 and *β-actin*, respectively.