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Antimicrobial activity of a serine proteinase inhibitor SPI*Pm*5 from the black tiger shrimp *Penaeus monodon*

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ABSTRACT

A two-domain Kazal-type serine proteinase inhibitor, SPI*Pm*5, from *Penaeus monodon* was studied. Its transcript was expressed in all tissues tested including the hemocytes, stomach, gill, lymphoid organ, muscle, intestine and heart albeit less in hepatopancreas and eyestalk. The expression of *SPIPm5* gene was also up-regulated by heat stress, white spot syndrome virus (WSSV) infection and yellow head virus (YHV) infection. Injection of recombinant rSPI*Pm*5 protein into normal shrimp to mimic heat stress condition did not have or had little stimulating effect on the expression of other immune genes: *crustinPm1*, *penaeidin3*, *penaeidin5*, *Hsp70*, *SPIPm2* and *SPIPm5*. Like some other proteinase inhibitors, the rSPI*Pm*5 could inhibit the hemolymph proPO activity. In survival experiments, the rSPI*Pm*5 could prolong the life of WSSV-infected shrimp similar to the effect of heat stress. The rSPI*Pm*5 also helped the YHV-, *Vibrio harveyi*- and *V. parahaemolyticus*-infected shrimp survive longer. The increased endurance against microbial infection was due to the inhibitory effects presumably activated by rSPI*Pm*5 on viral replication and bacterial growth but not the expression of antimicrobial peptides. Therefore, the SPI*Pm*5 plays an important role in shrimp innate immunity against the viral and bacterial infection.

1. Introduction

Invertebrates rely on innate immunity, which are cellular and humoral responses, to defense themselves against pathogenic invaders. The defense responses are phagocytosis, blood coagulation, melanization, etc. The humoral defense involves the soluble components such as antimicrobial peptides (AMPs), proteinase inhibitors and cytokine-like protein [1,2]. The proteinase inhibitors come into play in the innate immune responses because several immune response processes are proteinase-mediated [3]. Several types of proteinase inhibitors are found in living organisms such as serpins, Kunitz-type inhibitors, Kazal-type inhibitors, Bowman-Birk inhibitors, pacifastin, etc. [4].

The Kazal-type serine proteinase inhibitors (KPIs) are characterized by one or more inhibitory domains of about 40–60 amino acid residues. The domain structure is formed by three intra-domain disulfide cross-links among six conserved cysteine residues [5]. The specificity of proteinase inhibition is mainly determined by the P1 amino acid for the side chain of P1 amino acid can fit into a pocket within the active site of a cognate proteinase. The inhibition of proteinase is a competitive-type inhibition [3,5].

In crustacean, the KPIs are implicated in host defenses against the pathogen infection because their expression is changed in response to microbial infection. In Chinese shrimp, *Fenneropenaeus chinensis*, the *FcSPI* genes are up-regulated in response to bacterial and viral infection [6,7]. In freshwater crayfish, *Procambarus clarkii*, the expression of four KPIs, *hcPcSPI1-4*, are changed by microbial infection. The *hcPcSPI1* and *hcPcSPI2* also possess bacteriostatic activity against the *Bacillus subtilis* and *B. thuringiensis* [8,9]. In swimming crab, *Portunus trituberculatus*, one-domain KPI, *PtKPI*, has been identified from the hemocytes. The temporal expression of *PtKPI* is activated against bacterial and fungal challenge [10].

In black tiger shrimp, *Penaeus monodon*, there are at least 9 types of KPIs, SPI*Pm*1-9, identified from the EST database [11]. A five-domain SPI*Pm*2 is the most abundant KPI and involved in defense against pathogens [12,13]. Its domains inhibit subtilisin and elastase and possess bacteriostatic activity against *B. subtilis* [14]. The SPI*Pm*2 is produced mainly by the hemocytes, stored in the cytoplasmic granules and secreted into the circulation upon activation [15]. The expression of SPI*Pm2* is up-regulated after WSSV and YHV infection [16,17]. The two-domain SPI*Pm4* and *5* are identified from the hemocytes of heat-

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treated shrimp. They have proteinase inhibitory activity but lack bacteriostatic effect against bacteria. The SPI*Pm*4 inhibits subtilisin whereas the SPI*Pm*5 inhibits subtilisin and elastase. The expression of *SPIPm5* is up-regulated by heat stress [18].

In this study, the possible function of SPI*Pm*5 and its expression in response to pathogen infection were studied in detail. The effects of recombinant SPI*Pm*5 protein on the expression of antimicrobial peptides, on the proPO activating system in hemolymph, on the survival rate of viral and bacterial infected shrimp, on the bacterial counts in hemolymph of infected shrimp and on the AMPs gene expression were elucidated.

2. Materials and methods

2.1. Shrimp

The black tiger shrimp were purchased from a local farm. The shrimp were acclimatized in seawater at ambient temperature of 28 ± 1 °C for a few days before used in the experiment.

2.2. Tissue distribution

Nine tissues were collected from the normal shrimp: hemocytes, stomach, hepatopancreas, gill, lymphoid organ, muscle, eyestalk, intestine and heart. The total RNAs were extracted using Trizol reagent (Geneaid) following the manufacturer instruction. The cDNAs were synthesized with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). The expression of *SPIPm5* gene in various tissues was analyzed by RT-PCR. The PCR reaction mixture consisted of 1.25 unit Taq DNA polymerase (RBC), 10 μM dNTP mix, 10 μM each of forward and reverse primers and appropriately 1:10 diluted cDNA template. The PCR started with pre-denaturation at 95 °C for 3 min followed by 30 cycles for *SPIPm5* or 25 cycles for *EF-1α* of denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and ended with final extension at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis. The expression of *EF-1α* was used as an internal control.

2.3. SPIPm5 was up-regulated under heat stress

To confirm that the *SPIPm5* was up-regulated after the shrimp were exposed to heat stress [18], the shrimp were heat shocked at 33 °C in seawater for 1 h and reared at ambient temperature. The hemolymph was, then, collected from three shrimp each at 24 and 48 h for total RNA preparation. The expression of *SPIPm5* gene was investigated along with that of *Hsp70* gene by RT-PCR. The hemolymph from shrimp reared at ambient temperature were also collected as a control group. The experiment was done in triplicate. The total RNAs were extracted with Trizol reagent and the cDNAs were synthesized as described above. The expression of *SPIPm5* and *Hsp70* were amplified by RT-PCR using specific primers (Table 1). The gene expression was analyzed by agarose gel electrophoresis. The expression of *EF-1α* was used as an internal control.

2.4. Expression of SPIPm5 gene in response to WSSV and YHV infection

The normal shrimp were divided into three groups of 45 shrimp each. The challenged group was injected with 50 μL of WSSV suspension containing 16,950 copies of WSSV that caused 100% mortality within 4 days. Another challenged group was injected with YHV dilution that killed all the shrimp within 5 days. Normal saline (0.85% NaCl) injection was done for the control group. The hemolymph was collected from 9 shrimp at each time point for each group at 3, 6, 12, 24 and 48 h for total RNA extraction and cDNA synthesis. The experiment was done in triplicate.

Expression of *SPIPm5* was analyzed with qRT-PCR using primers RTSPIPm5F and RTSPIPm5R (Table 1). The qRT-PCR was carried out in a real-time thermal cycler (Bio-Rad Laboratories). The 10-μL reaction mixture consisted of 3 μL of 1:10 diluted cDNA template, 0.2 μM of each primer and 1 × QPCR Green Master Mix LRox (Biotechrabbit) containing DNA polymerase. The PCR amplification started with pre-denaturation at 95 °C for 8 min followed by 40 cycles of denaturation at 95 °C for 30 s, and extension at 60 °C for 3 s. The expression of *EF-1α* was used as an internal control. The relative expression of *SPIPm5* was calculated using a comparative method described by Pfaffl [19]. The mean data values were derived from triplicate amplifications and expressed as means ± standard deviations. Statistical analysis was done using the One-way ANOVA followed by the Duncan's new multiple range test. Data differences were considered statistically significant at $P < 0.05$.

2.5. Expression of rSPIPm5 protein

The recombinant SPI*Pm*5 protein (rSPI*Pm*5) was expressed using an *E. coli* expression system. An expression plasmid, pET28b_SPI*Pm*5 [18], was transformed into an *E. coli* BL21-CodonPlus(DE3). A transformant was cultured in LB broth at 37 °C and induced for the protein expression by 1 mM IPTG. The cells were collected by centrifugation at 2500 g, 4 °C for 20 min. The cell pellet was resuspended in phosphate buffer saline, pH 7.4 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and sonicated with Sonics Vibracell VCX750 to break the cells. The soluble rSPI*Pm*5 in the cell lysate supernatant was purified with Ni-NTA agarose column and eluted with imidazole. The purified protein was dialyzed against PBS. Its purity and its concentration were analyzed by SDS-PAGE [20] and determined by Bradford assay [21], respectively. The purified rSPI*Pm*5 protein was used as an antigen for raising mouse polyclonal antiserum at the Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus.

2.6. Effect of rSPIPm5 injection in shrimp

The normal shrimp were divided into two groups of 9 shrimp each. First group was injected with 0.5 nmol of rSPI*Pm*5 in 30 μL of PBS. The control group was injected with 30 μL of PBS. The experiment was done in triplicate. After the rSPI*Pm5* protein injection, the hemolymph was collected at 24 h for total RNA extraction and cDNA synthesis. The gene expression of *crustinPm1, penaeidin3, penaeidin5, SPIPm2, SPIPm5* and *Hsp70* were investigated by RT-PCR using specific primers (Table 1). The PCR products were analyzed by gel electrophoresis. The expression of *EF-1α* gene was used as an internal control.

2.7. Effect of SPIPm5 protein on the proPO activating system in shrimp hemolymph

Inhibitory activity of rSPI*Pm*5 on proPO activating system was investigated using a protocol described by Ponprateep et al. (2017) [22]. The hemolymph was collected from three normal shrimp and mixed with PBS at a ratio of 1:3. The protein content was determined by using Bradford assay [21]. The reactions were done by pre-incubating 200 μg protein of hemolymph with 0, 0.5, 1 and 2 nmol of rSPI*Pm*5. The PBS was used as a control. Then, 10 μL of 5 mg/mL of lipopolysaccharide (LPS) was added and incubated at 30 °C for 15 min. The reaction was adjusted to 150 μL with 50 mM Tris-HCl pH 8.0. Finally, the reaction was added 25 μL of 3 mg/mL L-3,4-dihydroxyphenylalanine (L-DOPA) at time zero to start the reaction. The proPO activity was monitored by measuring the A_{490} for 60 min with a microplate reader. The experiment was done in triplicate.

2.8. Effect of SPIPm5 on the survival of pathogen-infected shrimp

2.8.1. rSPIPm5 injection and heat stress against WSSV infection

The normal shrimp were divided into five groups of 30 shrimp each.