1 **Domain 2 of a Kazal serine proteinase inhibitor SPIPm2 from *Penaeus monodon***
2 **possesses antiviral activity against WSSV**

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27 **Abstract**

28 A 5-domain Kazal type serine proteinase inhibitor SPIPm2 from *Penaeus monodon* is
29 involved in innate immune defense against white spot syndrome virus (WSSV). To test which
30 domains were involved, the 5 domains of SPIPm2 were over-expressed and tested against
31 WSSV infection. By using hemocyte primary cell culture treated with each recombinant
32 SPIPm2 domain along with WSSV, the expression of WSSV early genes *ie1*, *WSV477* and late
33 gene *VP28* were substantially reduced as compared to other domains when the recombinant
34 domain 2, rSPIPm2D2, was used. Injecting the WSSV along with rSPIPm2D2 but not with
35 other domains caused delay in mortality rate of the infected shrimp. The results indicate that
36 the SPIPm2D2 possesses strong antiviral activity and, hence, contributes predominantly to the
37 antiviral activity of SPIPm2.

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50 **KEYWORDS:** Serine proteinase inhibitor; Kazal domain; *Penaeus monodon*; WSSV;

51 Shrimp survival

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53 **1. Introduction**

54 Many processes like food digestion, blood feeding, metamorphosis, tissue modeling,
55 blood coagulation, melanization, etc., in animal use proteinase cascades to activate and
56 augment the processes. These processes are needed to be regulated by proteinase inhibitors for
57 the proteinase activity is deleterious to the tissues nearby [1–5]. The proteinase inhibitors are
58 thus widespread in multicellular organisms. They are proteins with different structural and
59 working designs. For instance, a suicide inhibitor serpin inhibits its cognate proteinase by
60 covalently linking its cleavage peptide bond to the catalytic amino acid in the proteinase,
61 changing the overall structure of the proteinase-inhibitor complex and trapping the proteinase
62 in irreversible non-functional stage [6,7]. On the other hand, a group of cysteine-rich proteinase
63 inhibitors such as serine proteinase inhibitors of types Kazal, Kunitz, Bowman–Birk, etc.
64 inhibit their cognate proteinases by reversible competitive inhibition [1,8].

65 In arthropods, the Kazal-serine proteinase inhibitors (KPIs) are well known. It has been
66 shown that the KPIs are involved in host innate immune defense system against pathogenic
67 microorganisms. Some KPIs show inhibitory activity against bacterial and fungal proteinases
68 such as those isolated from *Galleria mellonella* [9], *Bombyx mori* [10] and *Fenneropenaeus*
69 *chinensis* [11]. Some KPIs possess antimicrobial activity like the KPIs from *Procambarus*
70 *clarkii* [12] and *Hydra magnipapillata* [13]. Some KPI genes from *Argopecten irradians* [14],
71 *Chlamys farreri* [15] and *Fenneropenaeus chinensis* [16] are up-regulated upon bacterial or
72 viral challenges implying that the KPI gene products are involved in the pathogen defense
73 system.

74 In this study, we are interested in a Kazal-serine proteinase inhibitor from black tiger
75 shrimp *Penaeus monodon*. Of the nine types of KPIs identified from the EST libraries of *P.*
76 *monodon*, the SPIPm2 is the most abundant [17]. The SPIPm2 consists of 5 Kazal domains
77 capable of inhibiting the proteinase subtilisin and elastase [18]. It also possesses bacteriostatic

78 activity against *Bacillus subtilis* [19]. The SPIPm2 is synthesized and stored in the hemocyte
79 granules waiting to be released upon trigger by pathogenic infection [20,21].

80 We have found that the expression of SPIPm2 is up-regulated in shrimp upon white
81 spot syndrome virus (WSSV) infection and the WSSV replication is suppressed by the SPIPm2
82 [20–22]. Since there are 5 Kazal domains in the SPIPm2, it is, then, interesting to find out
83 which Kazal domain is able to interfere with WSSV replication. By testing the individual Kazal
84 domains with WSSV-infected hemocyte cell culture and WSSV-infected shrimp, we have
85 shown that domain 2 of SPIPm2 possesses interfering effect on the WSSV replication.

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87 **2. Materials and Methods**

88 **2.1 Expression and purification of recombinant proteins**

89 The five Kazal domain proteins of SPIPm2 were prepared according to Donpudsa et al.,
90 2009 [19]. Briefly, an *E. coli* Rosetta(DE3)pLysS transformant containing each SPIPm2
91 domain expression plasmid, pSPIPm2-D1 to pSPIPm2-D5, was cultured under vigorous
92 shaking at 37 °C until the optical density at 600 nm reached 0.6 – 0.8. Then, the IPTG was
93 added to the final concentration of 1 mM and the culture was continued for additional 3 h. Cells
94 were harvested by centrifugation, resuspended in phosphate-buffered saline pH 7.4 (1× PBS;
95 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) and sonicated to break the
96 cells. The cell lysate was centrifuged at 8,000 rpm for 10 min at 4 °C to collect the supernatant.

97 The soluble recombinant protein in the supernatant was purified using a Ni-NTA
98 agarose column and eluted with the 1× PBS buffer pH 7.4 containing 500 mM imidazole.
99 Consequently, the fractions containing the eluted protein were dialyzed against the 1× PBS
100 buffer. The protein was traced using SDS-PAGE. The concentration of protein was determined
101 using the Bradford method [23]. The recombinant thioredoxin was produced by the same above

procedure from an expression vector pET-32a(+) and used as a control protein in the experiments described below.

2.2 Proteinase inhibition assays

The inhibitory activity of each domain against serine proteinases; trypsin (bovine pancreas, Sigma), subtilisin Carlsberg (*Bacillus licheniformis*, Sigma) and elastase (porcine pancreas, Pacific Science), was assayed using a procedure of Hergenhahn et al. (1987) [24] and Donpudsa et al. (2009) [19]. The reaction mixtures consisted of a two-fold serially diluted amount of a Kazal domain of SPIPm2; 50 mM Tris-HCl pH 8; 146.8 and 293.6 μ M of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (Sigma) for trypsin and subtilisin or 886.1 μ M of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma) for elastase; and 0.02, 0.04 and 0.08 μ M of subtilisin, trypsin and elastase, respectively, in a total volume of 100 μ l. The reactions were incubated at 30 °C for 15 min and then terminated by adding 50 μ l of 50% acetic acid. The *p*-nitroaniline formed was measured at the absorbance of 405 nm. The percentages of remaining activity were calculated and plotted against the molar ratios of inhibitory domain to proteinase.

2.3 Shrimp hemocyte primary cell culture

Shrimp hemocyte primary cell culture was prepared according to Ponprateep et al., 2011 [20]. Briefly, hemocyte cells from ten normal shrimp were collected using a modified Alsever's solution as an anticoagulant, pooled and resuspended in 2 $\times$ L15 medium supplemented with 20% fetal bovine serum (GIBCO). The cell suspension was aliquot into each well of the 24-well plate (1×10^6 cells/400 μ l/well) and incubated at 28 °C for 3 h for the cells to attach to the plate. The fresh culture medium of 200 μ l was replaced in each well every day.

2.4 Antiviral activity assay of SPIPm2 Kazal domains using hemocyte primary cell culture

In order to test the antiviral activity against WSSV, the purified WSSV (1.75×10^5 copies) was incubated with 10 μ M thioredoxin or 10 μ M each Kazal domain of SPIPm2 in 120 μ l 1 $\times$ PBS for 15 min before adding 100 μ l of the mixture into the hemocyte primary cell culture. The WSSV-infected hemocytes were collected at 24 hours post infection (hpi) and subjected to total RNA extraction using a FavorPrep™ Blood/Cultured Cell Total RNA Purification Mini Kit (Favorgen). The extracted total RNA was treated with RNase-free DNase I (Promega). The first strand cDNA synthesis was carried out using the First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's instruction. The synthesized cDNA was analyzed by RT-PCR

The gene specific primers, ie1-F (5' GACTCTACAAATCTCTTTGCCA 3') and ie1-R (5' CTACCTTTGCACCAATTGCTAG 3') for *ie1* gene, WSV477_RT-F (5' ATGTATATCTTCGTCGAAG 3') and WSV477_RT-R (5' TTATAAGAAATGTACAATCC 3') for *WSV477* gene, VP28_RT-F (5' TCACTCTTTCGGTCGTGTCG 3') and VP28_RT-R (5' CCACACACAAAGGTGCCAAC 3') for *VP28* gene of WSSV and EF-F (5'GGTGCTGGACAAGCTGAAGGC 3') and EF-R (5' CGTTCCGGTGATCATGTTCTTGATG 3') for *EF-1 α* gene, were used to amplify the gene products. The *EF-1 α* gene of shrimp was used as an internal control gene. The PCR reaction contains 1 $\times$ PCR buffer, 200 μ M dNTP, 0.2 μ M each specific primer, 1 unit Taq DNA polymerase (RBC) and appropriate amount of cDNA from the WSSV-infected hemocyte cells. The PCR condition began with an initial denaturation at 94 °C for 2 min followed by 36 cycles for *ie1*, 30 cycles for *VP28* or 35 cycles for *WSV477* of denaturation at 94 °C for 30 sec, annealing at 58 °C for *ie1* gene, 65 °C for *VP28* gene for 45 sec or 50 °C for 1 min for *WSV477*, elongation at 72 °C for 1 min and the final extension at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis.

Experiments were performed in triplicate for statistical analysis. The band intensities of PCR products were determined using the Genetools analysis software. The expression of *ie1*, *WSV477* and *VP28* genes was reported relative to that of the internal control, *EF-1 α* . The data were expressed as means $\pm$ standard deviations. The statistical significance of the data was evaluated using one-way ANOVA followed by post hoc test (Duncan's new multiple range test). Significant differences were accepted at $P < 0.05$.

2.5 Effect of the Kazal domains of SPIPm2 on the survival of WSSV-infected shrimp

Shrimp were divided into eight groups of ten shrimp. Each group was injected with 2.0 nmol thioredoxin, 2.0 nmol thioredoxin plus WSSV, 2.0 nmol of rSPIPm2D1-D5 plus WSSV and 2.0 nmol rSPIPm2 plus WSSV. The WSSV used had the LD₅₀ at 4 days. The shrimp mortality rate was observed for nine days. The experiment was repeated three times for statistical analysis. The data were analyzed and plotted using GraphPad Prism.

3. Results and discussion

Previously, a five-domain KPI namely SPIPm2 was identified from the hemocyte EST libraries of *P. monodon* [25]. Among the 9 KPIs identified from *P. monodon*, the SPIPm2 is the most abundant KPI in shrimp [17]. The proteinase inhibitory activity of SPIPm2 and its domains has been studied [18,19]. Each domain of SPIPm2 presents different proteinase inhibitory activity according to the P₁ amino acid (Fig. 1). Besides having proteinase inhibitory activity, the SPIPm2 is also involved in innate immunity for its expression is up-regulated in response to WSSV infection. Moreover, the recombinant SPIPm2 (rSPIPm2) is able to inhibit the expression of WSSV envelope protein gene *VP28* which can be deemed as the reduction of WSSV replication and helps prolong the lives of WSSV-infected shrimp [22].

Since the SPIPm2 possesses 5 Kazal domains, it is, then, interesting whether the domains of SPIPm2 can act upon the WSSV. In order to investigate the effect of each Kazal

domain of SPIPm2 on WSSV infection, the 5 recombinant domain proteins, rSPIPm2D1 to rSPIPm2D5, were over-expressed in an *E. coli* expression system. Each domain protein was tagged with thioredoxin and His tag at its N-terminus and C-terminus, respectively [19]. The thioredoxin-tagged domain proteins were produced as soluble proteins. The domain proteins were purified using the Ni-NTA agarose column and analyzed using 15% SDS-PAGE. The result in Fig. 2 shows only one major band for each rSPIPm2 domain protein. For a Cys-rich protein, it is normal to observe small amount of dimer and trimer. The molecular weight of each domain protein is estimated to be about 20 kDa.

The purified rSPIPm2 domain proteins were assayed for their proteinase inhibitory activity to make certain that they were active even in the form of fusion protein with thioredoxin. They were tested against trypsin, chymotrypsin, subtilisin and elastase. It was found that the inhibitory activity was comparable to that of the non-fusion domain protein (Fig. 3) [19]. The rSPIPm2D1 was inactive not only with subtilisin but also elastase and trypsin (data not shown for elastase and trypsin) (Fig. 3 B). The rSPIPm2D2 inhibited elastase and subtilisin but not trypsin. The rSPIPm2D3 and rSPIPm2D5 inhibited only subtilisin. The rSPIPm2D4 interfered with the activity of trypsin and subtilisin. We do not consider the rSPIPm2D4 to have inhibitory activity of proteinase inhibitor because its action is a lot less potent even at the mole ratio of rSPIPm2D4 to proteinase as high as 50 to 1.

The domain proteins were, then, tested against WSSV using the shrimp hemocyte primary cell culture to see if there was any antiviral activity. In this experiment, the WSSV was incubating with each domain protein before adding into the cell culture. After 24 h post infection, the hemocyte total RNA was extracted for expression analysis of early viral protein genes *iel*, *WSV477* and a late gene *VP28*. If the expression of *iel*, *WSV477* and *VP28* was reduced, it meant that the replication of WSSV would be negatively affected too. The RT-PCR results revealed that the expression levels of early genes *iel* and *WSV477* were dramatically

reduced by rSPIPm2D2 as compared to the thioredoxin control by about 80% and 90%, respectively, and that of VP28 was reduced by only 50% (Fig. 4). The rSPIPm2D1, D3, D4 and D5, on the other hand, showed considerably less antiviral activity.

If any of the rSPIPm2 domain protein is capable of repressing the expression of *ie1*, WSV477 and VP28, we would expect that the said rSPIPm2 domain protein should be able to more or less negatively affect the replication of WSSV and reduce the survival rate of WSSV-infected shrimp. The survival rate assay was carried out to investigate the survival of shrimp injected with a mixture of WSSV and each rSPIPm2 domain protein. Each rSPIPm2 domain protein was mixed with the WSSV and injected into the shrimp. The intact rSPIPm2 was tested too for comparison. The survival was recorded and plotted against time as shown in Fig. 5. Without WSSV infection, the mortality of un-infected control was minimal. In the presence of rSPIPm2D1 to D5 except rSPIPm2D2, the survival rate of infected shrimp was in close vicinity having the LD₅₀ at 4 days. With rSPIPm2D2, the LD₅₀ was delayed to 5 days. The intact rSPIPm2 made it to 6 days. Though the rSPIPm2D2 was able to prolong the mortality rate of WSSV-infected shrimp, its effect was still less than that of the intact rSPIPm2. Thus, all the domains were actually required for efficient antiviral activity.

Since the rSPIPm2D2 and intact rSPIPm2 possess both proteinase inhibitory activity against elastase and subtilisin and the antiviral activity against WSSV, one may want to speculate that the SPIPm2 inhibits the cognate proteinases through its rSPIPm2D2 in the process of WSSV infection and, thereby, interferes with the viral replication. However, there is no evidence so far that there is any connection between the proteinase inhibitory activity and the antiviral activity. Nevertheless, this study provides the evidence that the antiviral activity of SPIPm2 is very likely exerted mainly through its SPIPm2D2 domain. How the SPIPm2D2 acts to hinder the WSSV remains to be elucidated.

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Figure legends

Fig. 1. Amino acid sequences of the five Kazal domains of SPIPm2. The sequence in each Kazal domain starts from the first cysteine residue to the sixth one. The conserved cysteine residues are shaded green. The P1 amino acids are in bolded letters. The P2, P1, P1' and P2' are indicated above the Kazal domain sequences. The asterisks, “.” and “:” indicate similarity among the amino acid sequences.

Fig. 2. A 15% SDS-PAGE of purified rSPIPm2 domain proteins. Lane M is the size marker. Lanes 1–5 are rSPIPm2D1–rSPIPm2D5, respectively.

Fig. 3. Proteinase inhibition assay of the rSPIPm2 domain proteins. Recombinant domain proteins D1 (✕), D2 (■), D3 (●), D4 (▲) and D5 (◆) were assayed for its inhibitory activity against elastase, trypsin and subtilisin at various mole ratios of inhibitor to proteinase. (A) The inhibition of elastase and trypsin of domain proteins D2 and D4, respectively. (B) The inhibition of subtilisin of domain proteins D1 to D5.

Fig. 4. Effect of rSPIPm2 domain proteins on the expression of WSSV early gene *ie1*, *WSV477* and late envelope protein gene *VP28*. (A) The WSSV was incubated with thioredoxin (Trx), rSPIPm2 or each rSPIPm2 domain protein (D1-D5) for 15 min before adding into the shrimp hemocyte primary cell culture. After 24 h post infection, the hemocyte cells were collected and processed for RT-PCR analysis of *ie1*, *WSV477* and *VP28* gene expression. The expression of *EF-1α* was used as an internal control. (B) The RT-PCR results were done in triplicate and scanned for the band intensity. The results were normalized relative to those of *EF-1α* and plotted as a bar graph showing the means with standard deviations. The statistical significance of the data was evaluated using one-way ANOVA followed by post hoc test (Duncan's new

multiple range test). Significant differences were accepted at $P < 0.05$ indicating by different letters within each gene.

Fig. 5. Effect of each recombinant Kazal domain protein of *SPIPm2* on the survival of WSSV-infected shrimp. Eight groups of ten shrimp were injected with thioredoxin alone (pink circle), thioredoxin plus WSSV (brown square), each recombinant *SPIPm2* domain protein plus WSSV (D1, dark gray triangle; D2, blue inverted triangle; D3, dark green diamond; D4, violet circle; D5, black square) and *SPIPm2* plus WSSV (green triangle). The shrimp mortality was recorded for nine days. The experiment was repeated three times. The data were plotted using GraphPad Prism as means $\pm$ standard deviations.

Fig 1

Fig 2

Fig 3

A. Elastase and trypsin inhibition

B. Subtilisin inhibition

Fig 4

Fig 5