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A five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon* and its inhibitory activities

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Abstract

A novel five-domain Kazal-type serine proteinase inhibitor, SPI*Pm*2, identified from the hemocyte cDNA library of black tiger shrimp *Penaeus monodon* was successfully expressed in the *Escherichia coli* expression system. The expressed recombinant SPI*Pm*2 (rSPI*Pm*2) as inclusion bodies was solubilized with a sodium carbonate buffer, pH10, and purified by gel filtration chromatography. The molecular mass of rSPI*Pm*2 was determined using MALDI-TOF mass spectrometry to be 29.065 kDa. The inhibitory activities of rSPI*Pm*2 were tested against trypsin, α-chymotrypsin, subtilisin and elastase. The inhibitor exhibited potent inhibitory activities against subtilisin and elastase, weak inhibitory activity against trypsin, and did not inhibit chymotrypsin. Tight-binding inhibition assay suggested that the molar ratios of SPI*Pm*2 to subtilisin and elastase were 1:2 and 1:1, respectively. The inhibition against subtilisin and elastase was a competitive type with inhibition constants (K_i) of 0.52 and 3.27 nM, respectively. The inhibitory activity of SPI*Pm*2 against subtilisin implies that, in shrimp, it may function as a defense component against proteinases from pathogenic bacteria but the elastase inhibitory function is not known.

Keywords: *Penaeus monodon*; Black tiger shrimp; Serine proteinase inhibitor; Kazal domain; Hemocyte

1. Introduction

Serine proteinase inhibitors (SPIs) are found widely in all multicellular organisms and play crucial roles in regulating many biological processes in which proteinases are involved by limiting the level and extent of proteinase activity in the processes [1]. The proteinase cascades in blood circulation, the blood clotting system, for example, is well regulated to prevent excessive clotting and digestion of the surrounding proteinacious tissues. In arthropods, proteinase inhibitor systems are important for metamorphosis [2,3] and in the host defense systems involving blood coagulation, prophenoloxidase cascades or cytokine activation, and selective digestion of various pathogens [4]. Such SPIs are members of established inhibitor families, namely, Kazal, Kunitz, serpin and α-macroglobulin families [4–6].

Many Kazal serine proteinase inhibitors from several multicellular organisms have been identified and characterized, for example in bird eggs, in mammalian tissues [1], in the blood-sucking insects *Rhodnius prolixus* [7], and *Dipetalogaster maximus*

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[8], etc. The Kazal inhibitors may contain one or more inhibitory domains. Several inhibitory domains are common in ovomucoid and ovoinhibitors from birds [9,10]. Potent thrombin-specific SPIs, namely rhodniin and dipetalgastin containing two and three Kazal domains have been isolated and characterized in the blood-sucking insects *R. proxilus* and *D. maximus*, respectively. In crustacean, the four domain Kazal-type inhibitors of crayfish, *Pacifastacus leniusculus*, has been characterized [11]. Recently a cDNA clone coding for a four-domain Kazal protein was isolated from a hemocyte cDNA library of the Pacific white shrimp, *Litopenaeus vannamei* [12]. Different Kazal inhibitors may arise from alternate splicing of the same gene, for example alternate splicing of KAZ1 results in several Kazal-type SPIs in *Drosophila* [13].

In previous studies, the expressed sequence tag (EST) libraries from the hemocytes of normal and *Vibrio harveyi*—infected black tiger shrimp *Penaeus monodon* were generated in order to identify genes associated with shrimp immunity [14]. Two types of Kazal-type SPIs, the four- and five-domain Kazal inhibitors, were identified. The latter was chosen for further investigation because it contained variations in the inhibitory domains. Its tissue-specific expression was studied. The inhibitor was expressed in *E. coli* and the inhibitory activity of the crude protein against proteinases was tested [15]. Owing to the low expression level of the inhibitor, which limited our study, we used another *E. coli* expression system and successfully expressed enough protein for further purification, inhibitory activity assay and kinetic studies.

2. Materials and methods

2.1. Bacterial strains

E. coli Rosetta(DE3)pLysS (Novagen), genotype; F^{-}*ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm lacY1* (DE3) pRARE (Cm^R)

E. coli XL1 blue, genotype; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F *proAB lacI*^{q} *ZΔM15 Tn10* (Tet^R)]

2.2. Construction of the expression plasmid

The 5′-terminal truncated serine proteinase inhibitor *Pm*2 (SPI*Pm*2) gene corresponding to the mature SPI*Pm*2 without signal peptide was constructed by polymerase chain reaction (PCR). A plasmid pSPI*Pm*2 [14], a pBlueScript SK plasmid containing the SPI gene, was used as a template. The primers used for the PCR were forward primer *Bam*HI–SPI*Pm*2-F, 5′-CCATGGATCCGGGC-TACGGAAAAGGGGGGAAAATCC-3′ and reverse primer *Sal*I-SPI*Pm*2-R, 5′-ATGGTCGAC-TAGGTACAGTCTGCGACCACAGATTCC-3′ as described in Jarasrassamee et al. [14]. The *Bam*HI–SPI*Pm*2-F was designed to delete the putative signal sequence of the SPI*Pm*2. The included *Bam*HI and *Sal*I sites were for the in-frame cloning of the 764 bp SPI*Pm*2 gene fragment into the expression vector pET-22b(+) (Novagen) downstream of the *pelB* signal sequence and upstream of a His · Tag.

The PCR reaction was performed in a final volume of 30 μl containing 25 ng of DNA template, 0.45 μM of each primer, 0.2 mM of each dNTP and 0.45 units of *Pfu* polymerase (Promega). The PCR amplification was run for 35 cycles of 45 s at 95 °C, 60 s at 56 °C and 60 s at 72 °C. After phenol–chloroform extraction and ethanol precipitation, the amplified SPI*Pm*2 gene was tailed with an adenine nucleotide and ligated to the pGEM®-T Easy vector (Promega). The resulting clone, pSPI*Pm*2-NS1, was isolated. The 755 bp *Bam*HI–*Sal*I fragment was further subcloned into the *Bam*HI–*Sal*I digested pET-22b(+) expression vector. The expression clone was selected and named pSPI*Pm*2-NS2. DNA sequencing was performed to confirm the correct junction between the vector and the inserted DNA as well as the sequence of the SPI*Pm*2 gene.

2.3. Expression of recombinant serine proteinase inhibitor

The recombinant pSPI-NS2 was transformed into an expression host, *E. coli* strain Rosetta(DE3)-pLysS (Novagen). Eighty microliters of glycerol stock of the transformant was inoculated into 2 ml LB broth containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol, and incubated with shaking at 37 °C until the OD_{600} reached 0.6–1.0. The cell suspension was inoculated into 50 ml LB medium and incubated with shaking at 37 °C until the OD_{600} reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was then added to a final concentration of 1 mM for the induction of expression. The culture was incubated further with shaking for an appropriate time. Aliquots of 1-ml culture at 0–4 h after induction were collected. Cells were harvested by

centrifugation and subjected to analysis. The protein concentration was determined according to Bradford [16] using bovine serum albumin as a standard. Expression of the recombinant SPI*Pm*2 (rSPI*Pm*2) was examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the cell lysate.

2.4. Analysis of the recombinant protein by SDS-polyacrylamide gel electrophoresis and Western blotting

The serine proteinase inhibitor samples were analyzed using 12% SDS-PAGE. The gels were stained with Coomassie brilliant blue R250.

Western blot analysis was carried out to confirm the identity of the rSPI*Pm*2. The proteins in the gel slab were blotted onto a nitrocellulose membrane. The rSPI*Pm*2 was detected by using anti-His antibody, horseradish peroxidase-tagged secondary antibody and immunochemical staining using diaminobenzidine as substrate according to the manufacturer's instructions (Qiagen).

2.5. Preparation of the recombinant serine proteinase inhibitor

After 3 h of IPTG induction, the cell pellet was collected by centrifugation at 6000*g* for 10 min, frozen completely at −80 °C, thawed at room temperature and resuspended by pipetting up and down in a lysis buffer (50 mM Tris-HCl, pH8, 5% glycerol and 50 mM NaCl). The suspension was shaken for an hour at room temperature, sonicated with a Bransonic 32 (Bandelin) for 4 min and centrifuged at 10,000*g* 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, pH10, at room temperature overnight. The insoluble material was removed by centrifugation.

2.6. Gel filtration column chromatography

The alkali-solubilized rSPI*Pm*2 was purified using Sephadex G-100 gel filtration column chromatography (Pharmacia). The protein sample was loaded into a 2 × 60 cm column, equilibrated with the elution buffer (50 mM sodium carbonate buffer, pH10) at a flow rate of 20 ml/h. Fractions of 3 ml were collected and monitored for protein by measuring the absorbance at 280 nm as well as the SDS-PAGE and SPI activity assay. The fractions showing the rSPI*Pm*2 band in SDS-PAGE and SPI inhibitory activity were pooled and concentrated for further analysis. The gel filtration column was calibrated with blue dextran 2000 (void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen A (25 kDa), myoglobin (17.5 kDa), cytochrome C (12.5 kDa), and potassium dichromate (total volume) for the estimation of the molecular weight of the rSPI*Pm*2.

2.7. MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry was used for an accurate determination of the molecular mass of the rSPI*Pm*2. It was performed in the commercial facility of the Proteomic Service Center, Bioservice Unit (BSU) (BIOTEC, Pathumthani, Thailand).

2.8. Tight-binding inhibition assay

The inhibitory activity of the rSPI*Pm*2 towards serine proteinases; trypsin (bovine pancreas, Sigma), α-chymotrypsin (type II bovine pancreas, Sigma), subtilisin Carlsberg (*Bacillus licheniformis*, Sigma) and elastase (porcine pancreas, Pacific Science), was assayed using a procedure of Hergenhahn et al. [17]. The reaction mixture consisted of 50 mM Tris-HCl, pH8; 146.8 and 293.6 μM of the chromogenic substrate, *N*-benzoyl–Phe–Val–Arg–*p*-nitroanilide (Sigma), for trypsin and subtilisin, respectively, 147.3 μM of *N*-succinyl–Ala–Ala–Pro–Phe–*p*-nitroanilide (Sigma) for chymotrypsin, or 443.1 μM of *N*-succinyl–Ala–Ala–Ala–*p*-nitroanilide (Sigma) for elastase; and 0.010, 0.005, 0.003 and 0.039 μM of subtilisin, trypsin, chymotrypsin and elastase, respectively, in a total volume of 100 μl. The proteinase was incubated with 0, 0.026, 0.052, 0.130, 0.207 and 0.413 μM of serine proteinase inhibitor. The reaction was incubated at 30 °C for 15 min and then terminated by adding 50 μl of 50% acetic acid. The absorbance of *p*-nitroaniline formed was measured at 410 nm. The remaining activity was calculated and plotted against the molar ratios of inhibitor to proteinases.

2.9. Kinetics of serine proteinase inhibition

The experiment was composed of four sets of reactions in which each set consisted of six concentrations of substrate in the presence of fixed amounts of serine proteinase and rSPI*Pm*2, and

three different concentrations of inhibitor were used. For subtilisin: 9.2 nM of subtilisin; 0, 0.147, 0.294, 0.440, 0.587, 0.734 and 1.028 mM *N*-benzoyl–Phe–Val–Arg–*p*-nitroanilide; and 0, 0.67, 1.34 and 2.68 nM rSPI*Pm*2 were used. For elastase: 19.3 nM of elastase; 0, 0.443, 0.886, 1.772, 2.658, 3.544 and 4.431 mM *N*-succinyl–Ala–Ala–Ala–*p*-nitroanilide; and 0, 1.34, 2.68 and 5.36 nM rSPI*Pm*2 were used.

The reactions were made into a total volume of 100 μl using 50 mM Tris-HCl, pH8. The reactions were initiated by the addition of proteinase. After incubating at 30 °C for 15 min, they were arrested with 50 μl of 50% acetic acid. The absorbance of *p*-nitroaniline formed was measured at 410 nm. The amount of *p*-nitroaniline was calculated using a millimolar extinction coefficient of 8.8. The activity was calculated as nmole of *p*-nitroaniline/min. The activities were plotted against the concentrations of substrates as a substrate saturation curve and a Lineweaver–Burk plot. The apparent K_Ms at different concentrations of inhibitor and V_{max} were determined. The apparent K_Ms were replotted against the concentrations of inhibitor. The latter plotting was constructed for the calculation of inhibition constant (K_i).

3. Results

3.1. Construction of the rSPIPm2 expression plasmid

The SPI*Pm*2 (GenBank Accession BI018075), a cDNA clone consisting of five Kazal domains, was identified from the hemocyte cDNA libraries of *Vibrio harveyi*—infected black tiger shrimp *Penaeus monodon* [14]. It was chosen for expression in the *E. coli* Rosetta(DE3)pLysS expression system.

The SPI*Pm*2 consists of an open reading frame of 801 base pairs coding for a protein of 266 amino acid residues (Fig. 1A). By using a SignalP prediction server [18], a putative signal peptide of 18 amino acid residues was predicted with a cleavage site between amino acid Ser18 and Gly19. The two oligonucleotides were designed for a PCR amplification of the 764 bp SPI*Pm*2 gene fragment from the pSPI*Pm*2 such that the signal peptide was excluded and restriction sites *Bam*HI and *Sal*I were included respectively at the 5′ and 3′ ends of the gene for cloning into the appropriate vector. The PCR fragment was 3′-tailed with adenine nucleotide and first cloned into pGEM®-T easy vector resulting in a plasmid pSPI*Pm*2-NS1. The *Bam*HI–*Sal*I SPI*Pm*2 gene fragment was then cloned from pSPI*Pm*2-NS1 into *Bam*HI–*Sal*I digested pET-22b(+). The latter cloning resulted in the fusion of SPI*Pm*2 to the 5′ *pel*B signal sequence and 3′ His · Tag sequence and the recombinant plasmid was named pSPI*Pm*2-NS2 (Fig. 1B).

3.2. Expression of SPIPm2 in E. coli expression system

The expression of rSPI*Pm*2 from the recombinant plasmid pSPI*Pm*2-NS2 was carried out in an *E. coli* Rosetta(DE3)pLysS by means of induction using 1 mM IPTG. After induction for 0, 1, 2, 3 and 4 h, 1 ml-aliquots of cells were harvested by centrifugation, solubilized with the gel loading buffer, and analyzed by 12% SDS-PAGE. The Coomassie brilliant blue staining of the gels revealed an induction of approximately 31 kDa protein (Fig. 2A). The 31 kDa was detected after 2 h of induction and gradually increased over time. For convenience, a three-hour induction was used to prepare the recombinant protein for further characterization.

Since the SPI*Pm*2 gene was fused to the His · Tag at its C-terminus, the identity of 31 kDa protein could be confirmed by Western blot analysis using anti-His antibody along with a secondary antibody tagged with horseradish peroxidase (HRP) for immunochemical staining. The Western blot in Fig. 2B suggests that the induced 31 kDa protein was a rSPI*Pm*2. A high molecular weight protein band, whose size corresponded to the dimer of rSPI*Pm*2, was also detected. The dimer had probably arisen after sample preparation for SDS-PAGE from some random disulfide bridge formation of various cysteine residues in this particularly cysteine-rich protein.

It is well documented that expression of heterologous proteins in *E. coli* may invariably result in an aggregate, collectively called inclusion bodies, particularly those proteins with high cysteine content [19,20]. The rSPI*Pm*2 is no exception. When the cell pellet was lysed, centrifuged to separate the lysate and insoluble debris, and analyzed by SDS-PAGE, the induced 31 kDa protein band was observed mainly in the insoluble protein fraction (data not shown), probably accounting for more than 80% of the insoluble protein. The rSPI*Pm*2 was thus expressed and aggregated as inclusion bodies, probably in the periplasmic space.

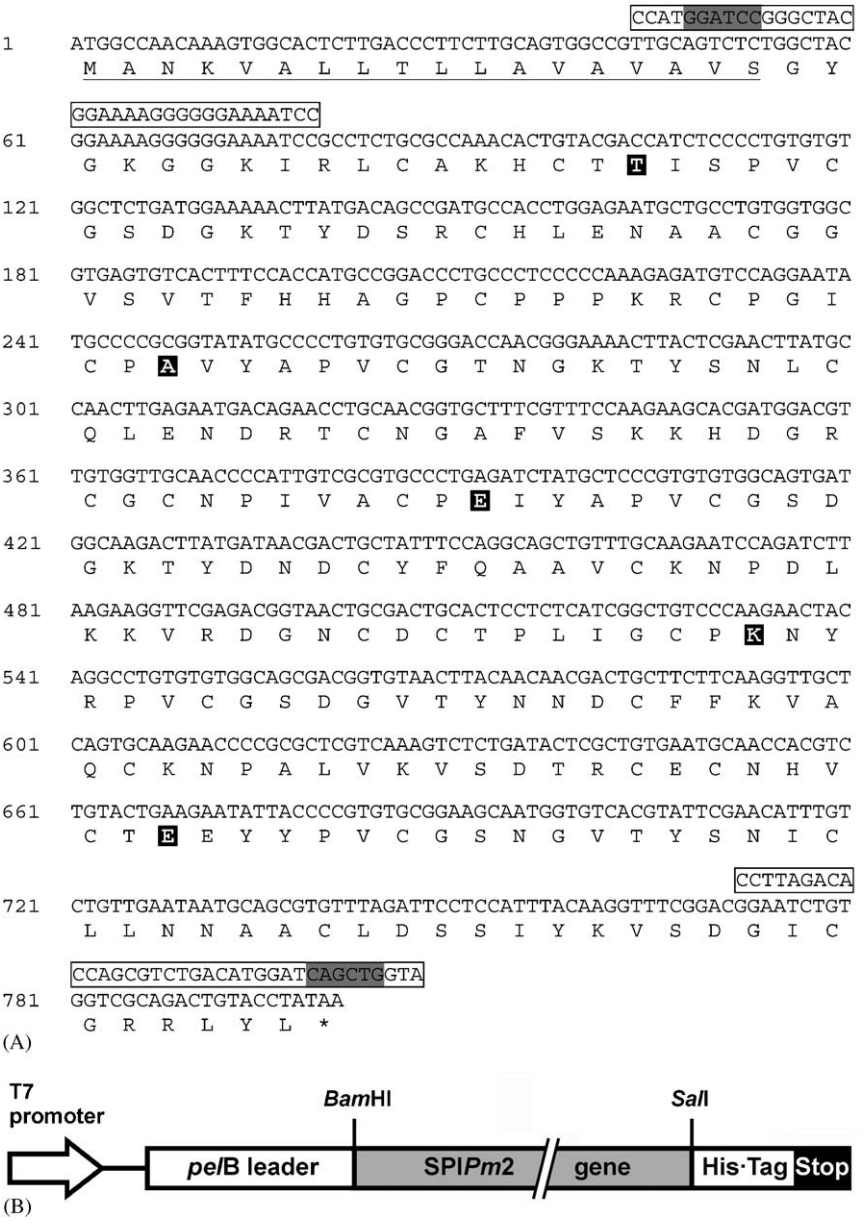

Fig. 1. The cloning of SPI*Pm*2 into the pET-22b(+) expression vector. (A) Nucleotide and amino acid sequences of the SPI*Pm*2. The putative signal peptide is underlined. The P1 residues are in the black boxes. The primers used for PCR amplification are boxed. The restriction sites in the primers are shaded. (B) Schematic representation of pSPI*Pm*2-NS2 in which the *Bam*HI–*Sal*I SPI*Pm*2 PCR fragment (shaded) was cloned in-frame into the expression vector downstream of the *pel*B leader sequence and upstream of the His · Tag.

3.3. Solubilization and purification of the recombinant protein

To purify the rSPI*Pm*2, the inclusion bodies were washed with 0.5 M NaCl and 1% Triton X-100 solution, then 0.5 M NaCl solution and finally distilled water. The pellet was then solubilized in an alkaline solution of 50 mM sodium carbonate, pH10. Preliminary assay of the solubilized rSPI*Pm*2 showed that the rSPI*Pm*2 exhibited strong inhibitory activity against subtilisin (data not shown). This inhibitory activity was then used to trace the protein upon purification.

The crude protein solubilized in 50 mM sodium carbonate, pH10, was purified by Sephadex G-100 column chromatography (Fig. 3A). The fractions

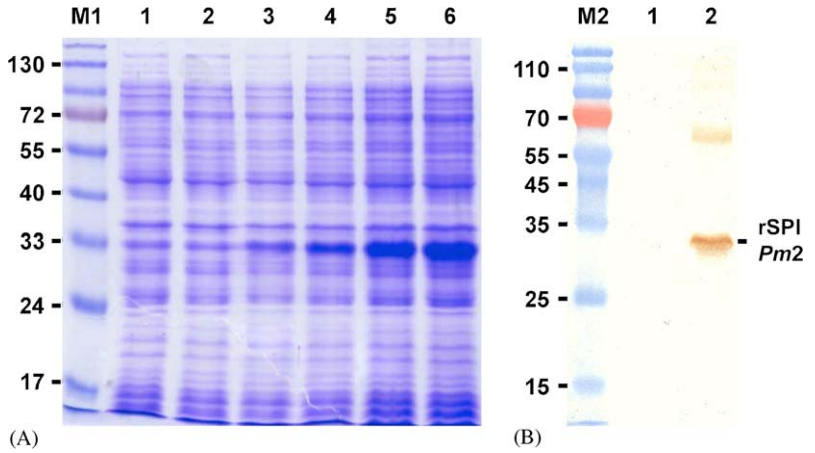

Fig. 2. SDS-PAGE and Western blot analysis of rSPI*Pm*2 expressed in *E. coli* Rosetta(DE3)pLysS. (A) The expression of rSPI*Pm*2 at 0–4 h after IPTG induction (lanes 2–6). Lane 1 is the *E. coli* lysate of cells containing parental plasmid pET-22b(+). (B) Identification of rSPI*Pm*2 as His · Tag protein. Lane 1 is the *E. coli* lysate of cells containing parental plasmid pET-22b(+). Lane 2 is the *E. coli* lysate of the cells containing the pSPI*Pm*2-NS2. Lanes M1 and M2 are 2 different prestained protein markers (Fermentas).

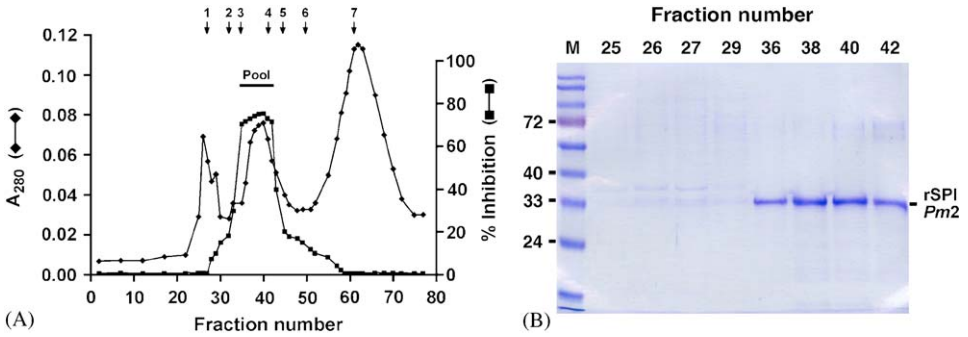

Fig. 3. Gel filtration chromatography of the rSPI*Pm*2. (A) The chromatographic profile of Sephadex G-100 gel filtration of crude rSPI*Pm*2. The horizontal bar indicates the fractions to be pooled. Numbers 1–7 represent the elution peaks of blue dextran, bovine serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen A (25 kDa), myoglobin (17.5 kDa), cytochrome c (12.5 kDa) and potassium dichromate, respectively. (B) The SDS-PAGE analysis of the eluted fractions showing the rSPI*Pm*2 band. Lane M is the prestained protein marker.

containing the SPI*Pm*2 as determined by inhibitory activity assay and SDS-PAGE were pooled and concentrated. Judging from the SDS-PAGE of the rSPI*Pm*2 fractions (Fig. 3B), the purified serine proteinase inhibitor was more than 95% pure with a small amount of high MW proteins. The purified SPI*Pm*2 was then used for further studies.

3.4. Molecular mass determination of the rSPIPm2

By comparing it to the standard proteins, the molecular weight of rSPI*Pm*2 was estimated by SDS-PAGE and gel filtration to be 31.8 and 31.4 kDa, respectively. However, the calculated molecular weight of rSPI*Pm*2 with or without the *pelB* signal sequence was 31.4 and 29.2 kDa, respectively. Since the SPI*Pm*2 was expressed as inclusion bodies, it was not certain that the *pelB* signal sequence had been removed. To clarify the matter, the molecular mass of the recombinant inhibitor was determined by using MALDI-TOF mass spectrometry as shown in Fig. 4. The spectrum of MALDI-TOF mass spectrometry revealed a major peak of the rSPI*Pm*2 with the actual molecular mass of 29.065 kDa indicating that the *pelB* signal sequence had indeed been removed from the protein. It further indicated that the rSPI*Pm*2 was aggregated in the periplasmic space of the host cells. There was a minor peak with a molecular mass of 27.8 kDa, which was faintly seen in SDS-PAGE.

There was also a small amount of high molecular weight proteins of 57–58 kDa. The molecular mass of rSPI*Pm*2 derived from the MALDI-TOF mass spectrometry was used subsequently in the calculation of the inhibitor concentration.

3.5. Tight-binding inhibition assay

To investigate the inhibitory activity of the inhibitor, tight-binding inhibition assay was carried out using increasing amounts of the purified rSPI*Pm*2 against trypsin, α-chymotrypsin, subtilisin and elastase. The remaining activities of proteinases were determined and plotted against the molar ratios of inhibitor to proteinases (Fig. 5). The rSPI*Pm*2 showed strong inhibitory activity against subtilisin and slightly lower activity against elastase but much lower activity against trypsin and it had no inhibitory activity against chymotrypsin. At a mole ratio of inhibitor to proteinase of 1:2, subtilisin was almost 90% inhibited suggesting that one molecule of inhibitor was able to inhibit two molecules of subtilisin. In other words, there were two inhibitory domains for subtilisin in rSPI*Pm*2. For elastase, the ratio of 1:1 was observed indicating only one elastase inhibitory domain in the rSPI*Pm*2. Since trypsin required more than ten-fold of inhibitor for the inhibition to be observed, it is obvious that the expected inhibitory domain for trypsin was not fully active as in other trypsin inhibitors.

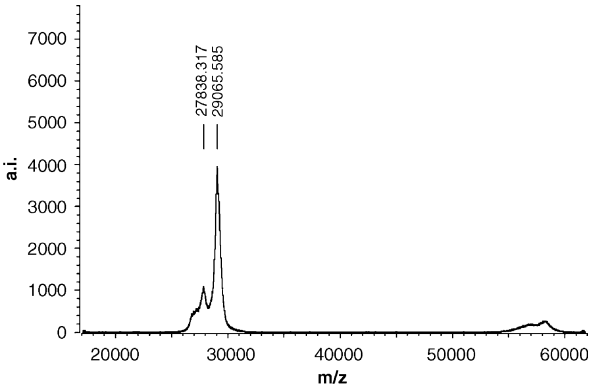

Fig. 4. The MALDI-TOF spectrum of the purified rSPI*Pm*2.

3.6. Determination of inhibition constant

To gain more insight into the inhibition of proteinases by SPI*Pm*2, kinetic studies of inhibition were carried out for subtilisin and elastase. The proteinases were assayed at various concentrations of substrate in the absence and presence of rSPI*Pm*2. The Lineweaver–Burk plots were constructed as shown in Fig. 6. The Lineweaver–Burk profiles were of a competitive type of inhibition. The K_Ms and V_{max}s were determined, respectively, to be 0.22×10^{-3} M and 1.25 nmole/min for the assay of subtilisin, and 1.73×10^{-3} M and 1.49 nmole/min for that of elastase. By replotting the apparent K_Ms against the inhibitor concentrations, the inhibition constants, K_is, were determined to be 0.52 and

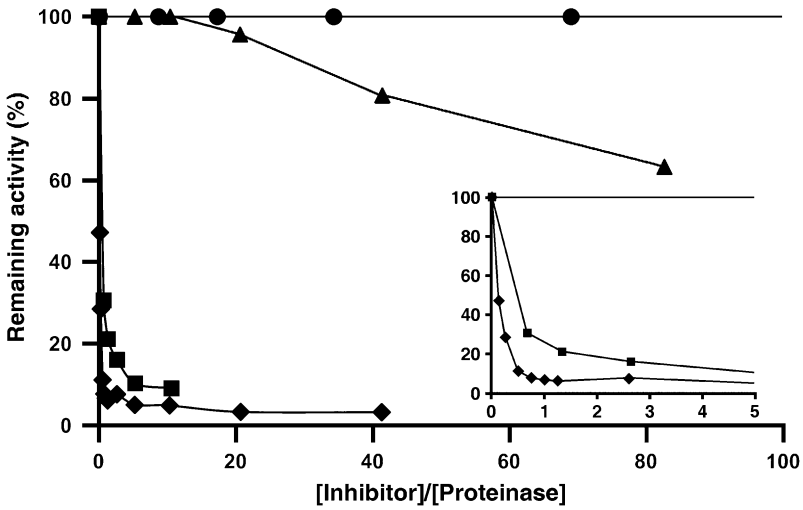

Fig. 5. The inhibitory affect of rSPI*Pm*2 on trypsin (▲), chymotrypsin (●), subtilisin (♦) and elastase (■). Inset is an expanded inhibitory profile of the same graph.

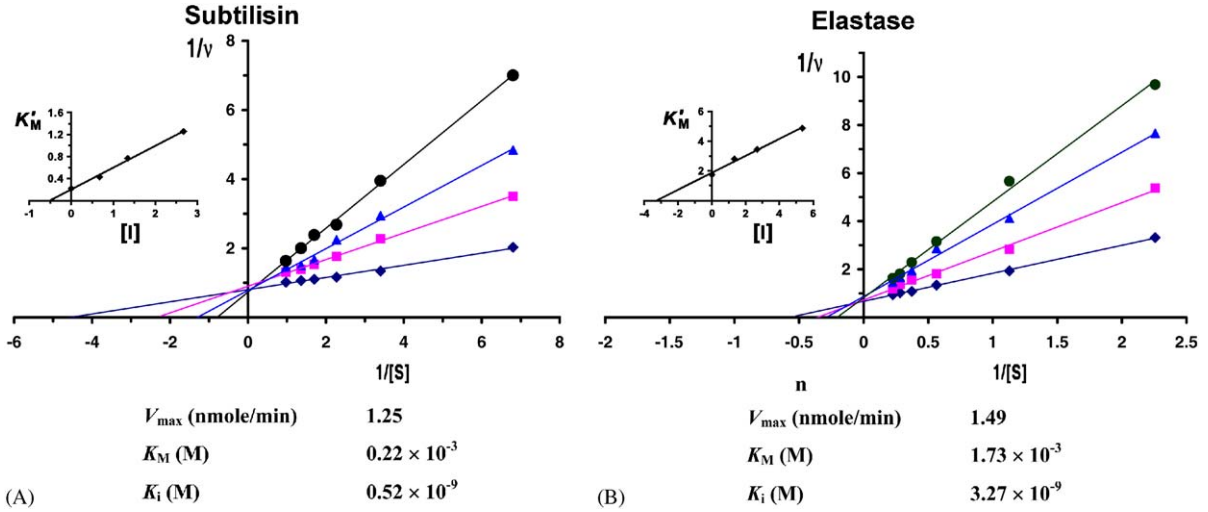

Fig. 6. Lineweaver–Burk plots of subtilisin (A) and elastase (B) activity assays at different concentrations of rSPI*Pm*2: 0 (♦), 0.67 (■), 1.34 (▲) and 2.68 nM (●). The plots of apparent K_Ms (K'_M) against the concentration of inhibitors are shown in the insets for the determination of K_is. The values of V_{max}s, K_Ms and K_is are indicated underneath the graphs.

3.27 nM for the inhibition of subtilisin and elastase, respectively (Fig. 6).

4. Discussion

Proteinase inhibitors are used by organisms to counteract proteinases for balances in several physiological functions, as responses to invading microbes, and for the invasion of hosts [5,21]. Among these, serine proteinase inhibitors (SPIs) are known to be widely distributed in living organisms to perform such functions. Several types of SPIs reside in the arthropod hemolymph, and their functions are believed to be inhibitors against proteinases from microorganisms and regulators of host-defense reactions involved in blood coagulation, prophenoloxidase activation or cytokine activation.

A number of SPIs possess one or more Kazal inhibitory domains and are grouped as the Kazal family of SPIs. The domain of Kazal-type serine proteinase inhibitors is a typical cysteine-rich domain, which is similar to the bovine pancreatic secretory trypsin inhibitor (PSTI) [22]. The inhibitory domain is made up of 50 to 60 amino acid residues with six-conserved cysteine residues forming three intra-domain disulfide bridges, which stabilize the domain [23]. The Kazal domain inhibits a proteinase in a substrate-like manner; it binds tightly to the active site of the proteinase. The inhibitory specificity of a domain is mainly dictated by the reactive P1 amino acid, which varies widely among different Kazal-type SPIs [1]. Other residues involved in enzyme inhibitor contact are also highly variable among avian ovomucoid third domain [24].

In crustaceans, three Kazal inhibitors, the four-domain Kazal-type inhibitor of crayfish, *Pacifastacus leniusculus* [11], the four-domain Kazal-type inhibitor of Pacific white shrimp, *Litopenaeus vannamei* [12], and the five Kazal-type inhibitors of black tiger shrimp, *P. monodon* [15], have been characterized. The five-domain Kazal SPI of *P. monodon*, SPI*Pm*2, was identified as an immune-related gene from the hemocyte EST libraries [14]. The five Kazal domains are in evidence when the amino acid sequences of the domains are aligned as compared to that of the four-domain Kazal inhibitor of the crayfish. The SPI*Pm*2 is of interesting for it contains five complete Kazal domains with diverse reactive P1 residues, and it is the first five-domain Kazal SPI reported in arthropods. Jarasrassamee et al. [15] attempted to express the protein using the *E. coli* expression system and the pTrcHis2C as vector. For unknown reasons, the recombinant protein was not over-expressed as expected and provided limited studies of its activities. In this study, by using a different *E. coli* expression system, pET-22b(+) and the *E. coli* strain Rosetta(DE3)pLysS, but a similar strategy to that of Jarasrassamee et al. [15], the SPI*Pm*2 was

successfully expressed. Upon IPTG induction, an approximate 31 kDa rSPI*Pm*2 was expressed as a major entity in the inclusion bodies.

Previously, we had used a buffered 6 M urea solution, a denaturing condition, to solubilize the inclusion bodies. The rSPI*Pm*2 was purified by using a Ni-NTA column. The inhibitor was then renatured by dialysis against the carbonate buffer, pH10. In our hands, the denaturing protocol was insufficient for efficient renaturation of the SPI*Pm*2 as the renatured protein was about 40% as active as that solubilized directly with the alkaline carbonate buffer (data not shown). The presence of several cysteine residues in the SPI*Pm*2 may render the polypeptide more difficult to refold correctly. The SPI*Pm*2, solubilized with alkaline buffer, was therefore used in this study.

By using MALDI-TOF mass spectrometry, the molecular mass of rSPI*Pm*2 was determined to be 29.065 kDa corresponding to the calculated molecular weight of the protein without the *pel*B signal sequence of 29.2 kDa.

The activity of rSPI*Pm*2 against proteinases was tested. It is generally well accepted that a major determinant for the Kazal inhibitory specificity is the P1 residue in the reactive site of SPI [1]. Therefore, the activity specificity of Kazal inhibitors may be predicted. Based on the various studies of the Kazal-type inhibitors, however, it is difficult to define the inhibitory specificity clearly. Generally, the SPIs with P1 arginine or lysine residues tend to inhibit trypsin [1]. The SPIs with P1 methionine residues from sea anemone and sheep lung are shown to inhibit elastase [25,26]. The SPI with leucine residue in the four-domain Kazal inhibitor from the crayfish inhibits chymotrypsin and subtilisin [11]. The SPIs with P1 threonine residues from *Bombyx mori* and *Galleria mellonella* inhibit subtilisin [27,28]. Though the five Kazal domains in SPI*Pm*2 contain P1 threonine, alanine, glutamate, lysine and glutamate residues, only the inhibitory activity against subtilisin and trypsin can be predicted.

The inhibitory assay of rSPI*Pm*2 against trypsin, chymotrypsin, subtilisin and elastase showed that the rSPI*Pm*2 was strongly active against subtilisin and elastase with the K_is of 0.52 and 3.27 nM, respectively, much less active against trypsin and not active against chymotrypsin. The extent of inhibition seemed to indicate that there were two subtilisin inhibitory domains and one elastase inhibitory domain as the binding ratios of inhibitor to subtilisin and elastase were approximately 1:2 and 1:1, respectively. The much lower activity against trypsin suggested that the inhibitory domain was a weak one. One might want to assign the two P1 glutamate residues to subtilisin inhibition and the P1 alanine or threonine residue to elastase inhibition. However, since the P1 threonine is found to be responsible to subtilisin inhibition [27,28], it is difficult then to assign definitely the function of two P1 glutamate residues. Accordingly, each complete Kazal domain of SPI*Pm*2 in *P. monodon* should be separately expressed in vitro and tested for proteinase inhibition.

The results of proteinase inhibition in this study are in contrast to the recent report by Jarasrasamee et al. [15] that the crude rSPI*Pm*2, expressed in *E. coli* using the pTrcHis2C as vector, has strong inhibitory activity against trypsin and chymotrypsin, weak activity against subtilisin and no activity against elastase. The discrepancy may arise from the fact that the previous work obtained considerably low yield of SPI with a lot of contaminated proteins. The contaminated proteins may in some ways interfere with the activity assays since the proteins themselves are also proteinase substrates. One may argue that in a prokaryotic system, the shrimp rSPI*Pm*2, which is a cysteine-rich protein with 30 cysteine residues, may have folded wrongly during biosynthesis. Thus, the activity of the recombinant protein may differ if different conformations are adopted. However, the rSPI*Pm*2 in this study was tested to have subtilisin and trypsin inhibitory activities as predicted, though the trypsin inhibitory activity was low, indicating that the over-expressed rSPI*Pm*2 was actually functional both in terms of structure and specificity. However, in the prokaryotic expression system, the possibility that the rSPI*Pm*2 was not perfectly folded could not be excluded.

The actual biological functions and target sites of the SPI*Pm*2 in hemocytes of *P. monodon* are not known. Since the blood cells are involved in immune defense against infection, the SPIs therein are likely to be components of the defense system and are secreted from the hemocytes in response to infection by pathogens or parasites. For example, a proteinase inhibitor of silkworm is active against proteinases from fungal pathogens [2]. Several purified proteinase inhibitors from crustaceans have also been shown to inhibit the pathogen proteinases [29]. In crayfish blood cells, the four-domain Kazal-type SPI blocks chymotrypsin and subtilisin and is thus

proposed as microbial proteinase inhibitor [11]. We have shown herein that the SPI*Pm*2 is a subtilisin–elastase inhibitor. Subtilisin is an alkaline proteinase secreted by Gram-positive bacteria or fungi whereas elastase-like serine proteinase is secreted by the blood cells. In human, neutrophil elastase is involved in tissue remodeling and blood coagulation at the sites of inflammation [30]. In crustaceans, the bacterial lipopolysaccharide activates a proteolytic cascade leading to the downstream coagulation reactions [31] but the nature of the proteinases has not yet been defined. The presence of inhibitory activity against subtilisin suggests to us that the SPI*Pm*2 may function as an inhibitor to the microbial proteinases. The function of elastase inhibitory domain, however, cannot be predicted.

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