Developmental and Comparative Immunology 33 (2009) 481–488

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

Domain inhibitory and bacteriostatic activities of the five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon*

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ARTICLE INFO

Article history:
Received 20 August 2008
Received in revised form 15 September 2008
Accepted 18 September 2008
Available online 16 October 2008

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

ABSTRACT

Serine proteinase inhibitors (SPIs) in multi-cellular organisms are important modulators of proteinase activities in various biological processes. A five-domain Kazal-type SPI SPI*Pm*2 from the black tiger shrimp *Penaeus monodon* is presumably involved in innate immune response. The SPI*Pm*2 with the domain P1 residues T, A, E, K and E was isolated from the hemocyte cDNA libraries and found to strongly inhibit subtilisin and elastase, and weakly inhibit trypsin. To unravel further the inhibitory activity of each domain, we subcloned, over-expressed and purified each individual SPI domain. Their inhibitory specificities against trypsin, subtilisin and elastase were determined. Domain 1 was found to be inactive. Domains 2, 3 and 5 inhibited subtilisin. Domain 2 inhibited also elastase. Domain 4 weakly inhibited subtilisin and trypsin. The intact SPI*Pm*2 inhibitor was found to possess bacteriostatic activity against the *Bacillus subtilis* but not the *Bacillus megaterium*, *Staphylococcus aureus*, *Vibrio harveyi* 639 and *Escherichia coli* JM109. Domains 2, 4 and 5 contributed to this bacteriostatic activity.

1. Introduction

Innate and adaptive immunity are important for multi-cellular organisms to protect themselves from the various potential pathogens. Invertebrates only have the innate immunity. The innate immune responses, carried out mostly by the hemocytes, can be divided into humoral and cellular defenses. These responses include phagocytosis, complement, antimicrobial peptides, coagulation and melanization [1,2]. Humoral defense usually involves components released from the hemocytes. These components include antimicrobial peptides, proteases and proteinase inhibitors [3].

The serine proteinase inhibitors (SPIs) are found widely in vertebrates, invertebrates and bacteria. They play important roles as modulators of several biological processes using proteinases, such as digestion, apoptosis, blood coagulation, prophenol oxidase activation, complement system, cellular remodeling, etc. [4–6]. They are also involved in metamorphosis [7], defense against invading organisms [8–11] or counter-defense the host protective proteases [12].

Among the at least 59 families of proteinase inhibitors, the Kazal, Kunitz, and pacifastin canonical inhibitors are relatively well characterized [13–15]. The Kazal-type SPIs are grouped into family I1. The Kazal-type SPIs typically contain one or more Kazal domains. Each domain of 50–60 amino acid residues has a characteristic three-dimensional structures derived from the formation of three intradomain disulfide bridges by six well-conserved cysteine residues [4,16]. The 'canonical' inhibition commences by tight binding of the SPI reactive site loop, into the active site of corresponding proteinase, competitively blocking and rendering the enzyme inactive [4]. The major inhibitory specificity determinant is the P1 amino acid residue which is the second amino acid residue after the second cysteine residue of the domain. A few other adjacent amino acid residues also influence the binding specificity [14,17].

Kazal-type SPIs are also found widely in all organisms. Besides those well-known Kazal-type SPIs like the LEKTI in human blood circulation [18] and the three-domain Kazal inhibitor in ovomucoid from chicken [19], more and more Kazal inhibitors have been identified in both vertebrates and invertebrates. For the recent examples in invertebrates, the SPIs containing four and five Kazal domains from *Litopenaeus vannamei* and *Penaeus monodon*, respectively, were identified from the hemocyte cDNA libraries [20,21]. A two-domain Kazal inhibitor, EPI1, from the oomycete plant pathogen *Phytophthora infestans*, was shown to inhibit subtilisin [12]. The 'nonclassical' Kazal-type elastase inhibitor was isolated from the sea anemone *Anemonia sulcata* [22]. The SPI called greglin specific for elastase/chymotrypsin from the ovary

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doi:10.1016/j.dci.2008.09.009

gland of the desert locust *Schistocerca gregaria* was characterized [23]. The mollusk Kazal-type SPI, AISPI, was identified from the bay scallop *Argopecten irradians* [24]. The three heat-tolerant human neutrophil elastase inhibitors, CmPI–I, CmPI–II and CmPI–III, were isolated from a marine snail *Cenchritis muricatus* [25]. The male reproductive tract specific two-domain Kazal-type SPI gene was identified in *Macrobrachium rosenbergii* [26]. Most recently, the 12-domain Kazal-type SPI gene was cloned from the Zhikong scallop *Chlamys farreri* [27].

In *P. monodon*, several Kazal proteinase inhibitors were identified from the expressed sequence tag (EST) database (http://pmonodon.biotec.or.th/) particularly the hemocyte libraries [28]. The most abundant SPI is the five Kazal-domain SPI*Pm*2. It was over-expressed and its activity has been studied [21]. The recombinant SPI*Pm*2 exhibits strong inhibitory activity against subtilisin and elastase, weak inhibitory activity against trypsin, and no activity against chymotrypsin. It turns out that the inhibitory specificities cannot be assigned to all Kazal SPI domain of the SPI*Pm*2. It is then interesting to find out the inhibitory specificity of each Kazal domain. In this study, each domain of SPI*Pm*2 was individually over-expressed and tested for their inhibitory specificities. As compared to the intact recombinant SPI*Pm*2 inhibitor, their growth inhibition on bacteria were also elucidated and reported herein.

2. Materials and methods

2.1. Construction of the expression plasmid

For the sake of protein purification, a modified expression vector pVR500 was constructed from an expression vector pET-32a(+) by deleting the His·Tag and S·Tag between *Msc*I and *Kpn*I sites. The only His·Tag left was at the 3′ side of the reading frame and used for the protein purification as described below. The pET-32a(+) was digested with *Msc*I and *Kpn*I, treated with T4 DNA polymerase to blunt the DNA ends and relegated. The resulting pVR500 was sequenced to confirm the correct construction. By using the expression vector pVR500, the cloned gene was fused with the thioredoxin Trx·Tag at the N-terminal side and His·Tag at the C-terminal end.

PCR primers were designed for the PCR amplification of SPI domains from the pSPI*Pm*2-NS2, a pET22b(+) containing the SPI*Pm*2 gene [21]. The forward and reverse primers contain *Nco*I and *Xho*I sites at their 5′ terminal ends, respectively. The restriction sites were for the cloning of SPI domain into the expression vector. The primer sequences and their annealing sites are depicted in Fig. 1 and Table 1. The PCR reaction was carried out 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 30 cycles of 45 s at 94 °C, 45 s at 55 °C and 60 s at 72 °C. The PCR product was gel-purified, digested with *Nco*I and *Xho*I and cloned into the pVR500 vector at the same restriction sites. The sequences of the domain clones were verified by DNA sequencing. Each recombinant plasmid was transformed into an *Escherichia coli* Rosetta(DE3)pLysS for over-production of the recombinant protein.

2.2. Expression and purification of the recombinant proteins

The *E. coli* Rosetta(DE3)pLysS transformants were cultured under vigorous shaking at 37 °C. When the optical density at 600 nm of the culture reached 0.6–0.8, the expression was induced by adding IPTG to the final concentration of 1 mM and the incubation was continued for additional 3 h. The expression of five recombinant proteins was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cells were harvested by centrifugation, resuspended in phosphate-buffered saline, pH 7.4 (1× PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and sonicated for 2–4 min. The cell lysate was centrifuged at 8000 rpm for 10 min at 4 °C to collect the supernatant. The soluble recombinant protein was purified using a Ni-NTA agarose column and eluted stepwise with the 1× PBS buffer pH 7.4 containing 500 mM imidazole. Consequently, the fractions containing the eluted protein were dialyzed against the enterokinase buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$) followed by incubation with enterokinase at 23 °C for 16 h in order to cleave the SPI domain away from the Trx·Tag. The final step was to purify the SPI domain using a Ni-NTA agarose column as described above. The purified SPI domain was dialyzed against 50 mM carbonate buffer, pH 10, and analyzed using SDS-PAGE. The concentration of eluted protein was determined using the Bradford method [29].

The intact SPI*Pm*2 was prepared according to Somprasong et al. [21].

2.3. SDS-PAGE and Western blot analysis

Standard 15% SDS-PAGE was used to analyze and trace the expressed recombinant proteins upon expression and purification. The protein bands were visualized by staining with Coomassie Brilliant Blue.

Western blot analysis was used to confirm the identity of the expressed recombinant proteins. The proteins separated by the SDS-PAGE were electro-transferred onto a nitrocellulose membrane (Bio-Rad) in a semi-dry electrophoretic transfer cell (Trans-blot SD, Bio-Rad) at 10 V for 30 min. The membrane was then washed twice with Tris-buffered saline (TBS; 10 mM Tris–HCl, 150 mM NaCl, pH 7.5) at room temperature for 10 min, and incubated in a blocking buffer (3% BSA in TBS buffer) at room temperature for 1 h. The membrane was subsequently washed twice with TBS containing 0.05% (v/v) Tween20 at room temperature for 10 min and incubated with anti-His antibodies (Qiagen) at room temperature for 1 h. After washing with TBS, the secondary antibodies conjugated with horseradish peroxidase were then added. The recombinant protein was visualized as a reddish-brown band with the HRP staining solution [18 mg diaminobenzine (DAB) dissolved in Tris–saline (9% (w/v) NaCl in 1 M Tris–HCl, pH 8.0)] and 30% hydrogen peroxide.

2.4. MALDI-TOF mass spectrometry

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

2.5. Inhibition assay

The inhibitory activity of each domain towards 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. [30]. The reaction mixture consisted of 50 mM Tris–HCl, pH 8; 146.8 and 293.6 μM of *N*-benzoyl–Phe–Val–Arg–*p*-nitroanilide (Sigma) for trypsin and subtilisin and 886.1 μM of *N*-succinyl–Ala–Ala–Ala–*p*-nitroanilide (Sigma) for elastase; and 0.02, 0.04 and 0.08 μM of subtilisin, trypsin and elastase, respectively, in a total