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Expression, purification and characterization of a three-domain Kazal-type inhibitor from silkworm pupae (*Bombyx mori*)

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Abstract

Serine protease inhibitors are essential for host physiological and immunological activities in insects. Analyzing the amino-acid sequence of a cDNA coding for a serine protease inhibitor in *Bombyx mori* (BmSPI), we found that BmSPI contained three homologous domains with a conserved sequence of C–X_3–C–X_9–C–X_6–Y–X_7–C–X_3–C–X_{11}–C similar to that of Kazal-type serine protease inhibitors, suggesting BmSPI as a new member of the Kazal-type serine protease inhibitor family. To characterize the three-domain Kazal-type inhibitor from silkworm pupae, the recombinant protein was expressed in *Escherichia coli* BL21 (DE3) Star. After purification with affinity and reversed-phase chromatographies, the recombinant BmSPI with a molecular mass of 33.642 Da was shown to be a specific subtilisin A inhibitor. Further studies indicated that the K_i value of the recombinant BmSPI was 3.35 nM and the inhibitor seemed to form a 1:1 complex with subtilisin A. This is a first description of the structure and characterization of Kazal-type inhibitor with three domains cloned from silkworm pupae, *B. mori*.

Keywords: *Bombyx mori*; Expression; Kazal-type; Purification; Serine protease inhibitor; Subtilisin inhibitor

1. Introduction

Serine protease inhibitors (SPIs), proteins first found in animal sera, exist in all multi-cellular organisms and play crucial roles in host physiological blood coagulation (Fuentes-Prior et al., 1997), development regulation (Konrad et al., 1998) and immunological functions (Gorman et al., 2000; Zhu et al., 2006), in which protease inhibitors are involved in regulating proteases activity (Laskowski and Kato, 1980). According to the primary sequence, disulfide bonds and three dimensional structure, they are at least classified into 18 protein families (Laskowski and Kato, 1980). However, the Kazal, Kunitz, α-macroglobulin, and Serpin families from insect and other arthropods, also play the same functions, thus, Kanost (1999) described them as powerful tools for understanding insect immunity at molecular level (Kanost, 1999).

As one of SPI families, Kazal-type SPIs, which contain multi inhibitory domains, have been widely identified and characterized to have diverse functions. For example, in crayfish blood cells, *Pacifastacus leniusculus*, the four-domain Kazal-type inhibitors have been characterized to inhibit chymotrypsin or subtilisin (Johansson et al., 1994); Dipetalgastin containing six Kazal domains has been identified to be a thrombin specific inhibitor in the blood-sucking insects *D. maximus* (Mende et al., 1999). Protease inhibitor 1 with four Kazal domains from *Toxoplasma gondii*, has been shown to inhibit trypsin and chymotrypsin (Morris et al., 2002). Recently SPIpm2 containing five Kazal domains from black tiger shrimp, *Penaeus monodon*, was detected to have potent inhibitory activities against subtilisin and elastase (Somprasong et al., 2006). cDNA clones coding for a four-domain and a six-domain Kazal protein were isolated from the cDNA library of the Pacific white shrimp, *Litopenaeus vannamei* and the bay scallops, *Argopecten irradians*, respectively (Jimenez-Vega and Vargas-Albores, 2005; Zhu et al., 2006).

But there has not been much work on protease inhibitors with Kazal domains in the silkworm, *Bombyx mori*, especially the multi-domain Kazal protease inhibitors. The silkworm, which is the major commercial silk producer and can produce medicinal protein as a bioreactor, play important roles as model insect. However, the silkworm always suffers from viral, bacterial, and protozoal infection. It was reported SPIs could

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prevent inappropriate degradation of silk proteins during their secretion (Kurioka et al., 1999) and regulate silkworm defense against pathogens or injury (Aso et al., 1994). Although SPIs were widely discovered and studied (Sasaki and Kobayashi, 1984), majority of which belong to the Kunitz-type SPIs, only several SPIs are single Kazal domain inhibitors, and their real functions are not very clear. Therefore, we first reported the expression, purification of the multi-domain Kazal protease inhibitor in hopes of having a further understanding of its characterizations and functions in *B. mori* in this paper.

2. Materials and methods

2.1. Construction of a recombinant plasmid

The BmSPI cDNA (Accession no. DN236893), isolated and cloned from the metaphase pupae previously by our laboratory (Zhang et al., in press), was used as a template to amplify interest fragment of the coding region by polymerase chain reaction (PCR). The PCR were performed with a TaqDNA Polymerase Kit (Promega, USA) and carried out at 94 °C for 5 min, followed by 30 cycles with denaturation 1 min at 94 °C, annealing 30 s at 60 °C, extension 1 min and 10 s at 72 °C and final extension step at 72 °C for 10 min. The oligonucleotides used in PCR were designed on the basis of the cDNA sequence of BmSPI and included restriction sites for *Bam*HI as well as *Xho*I (Promega, USA) for subcloning: sense primer was 5′-CTTAGGATCCCCATGTGTGGACTTG-3′, antisense primer was 5′-GTGTGCCTCGAGTTATTTGTCACCA-3′. The PCR products were purified after electrophoresis on 1% agarose gel using the PCR Rapid Purification Kit (BioDev-Tech, China). After digestion with *Bam*HI and *Xho*I, the interested parts were subcloned into the expression vector pGEX-5X-1 (AmershamBiosciences, USA) using T4 DNA ligase (Promega, USA) and transformed into *E. coli* TG1 cells (maintained in our laboratory) for screening purposes. A positive colony with the BmSPI gene in the plasmid was identified by double digestion of the plasmid, followed by analysis on 1% agarose gel electrophoresis and was subsequently sequenced by ABI Prism 3100-A.

2.2. Sequence analysis

The similarity analysis of nucleotide and protein sequence was carried out at GenBank using BLASTN (in the EST_others database) and BLASTP (in all non-redundant databases) algorithms. The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/). Multiple alignments of the Kazal domains were conducted using the program CLUSTAL-W of Bioedit software. Protein conformation was modeled by the SWISS-MODEL (http://swissmodel.expasy.org/) and viewed in the Swiss Pdb Viewer (Schwede et al., 2003).

2.3. Expression and purification of BmSPI

The expression vector pGEX-5X-1 was transformed in *E. coli* BL21 (DE3) Star cells (maintained in our laboratory) for expressing purposes. Bacterial expression cultures were incubated at 37 °C in LB medium containing ampicillin (50 μg/mL) until an A_{600} of 0.6 was reached. Recombinant protein expression was induced by the addition of IPTG (Sanland-chem, USA) to a final concentration of 0.5 mM. Following a 5 h incubation at 25 °C, bacteria were harvested by centrifugation at 10,000 ×*g* for 10 min at 4 °C, and the pellets were frozen at −20 °C.

Frozen bacterial pellets were thawed and resuspended in 25 mL of lysis buffer (1×PBS, 0.1% Triton X-100, 1 mM PMSF, pH 8.0), after addition of a 10 g lysozyme (sigma, USA) per 1 g bacteria, then incubated at 4 °C for 45 min. The bacteria were lysed by pulsed sonication with cell disruptor UP200H (Dr.Heilscher, Germany) for 20 min on ice. The lysates were centrifuged at 14,000 ×*g* for 30 min at 4 °C to remove cell debris and other particles. The supernatant was collected and filtered through a 0.45 μm filter (Millipore, USA). The solution was loaded to the 1-mL GSTrap FF column at a flow rate of 0.3 mL/min, which was equilibrated with the ice-cold binding buffer (1×PBS, pH 7.3) to the baseline. The column was washed with 10 volumes of binding buffer at a flow rate of 1 mL/min to remove unbound proteins. GST-BmSPI was eluted from the column at 4 °C with 5 vol. of elution buffer (50 mM tris-HCl, pH 8.0, containing 10 mM reduced glutathione). The eluted GST-BmSPI solution was subsequently ultrafiltrated (Microcon YM-10, Millipore Corp) to reduce the volume to 3 mL, remove the free glutathione and desalt the sample. The presence and purity of eluted GST-BmSPI was evaluated by SDS-PAGE using 12% SDS gels.

The solution containing GST-BmSPI protein was dialyzed against 400 volumes of Factor Xa cleavage buffer (50 mM tris-HCl, 100 mM NaCl, and 5 mM $CaCl_2$, pH8.0) for 24 h. For cleavage of BmSPI from GST tag, the dialyzed GST-BmSPI fusion protein solution was incubated with Factor Xa cleavage buffer plus Factor Xa (10 U/mg) for 16 h at 22 °C. After digestion, the protein solution was loaded to a GSTrap FF column, pre-equilibrated with cleavage buffer, to purify recombinant BmSPI by removing GST tag and undigested fusion protein.

The cleaved protein solution was redissolved in 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid/water and injected onto a μRPC C2/C18 reverse-phase HPLC column (AmershamBiosciences, USA) equilibrated with 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 1.0 mL/min. The concentration of acetonitrile in the elution buffer was raised from 5% to 95% (v/v) with a linear gradient for 45 min. Absorbance was monitored at 214 nm and fractions were collected. The eluted BmSPI was freeze-dried with an alpha 2–4 Freeze Dryer (Christ, Germany) then stored at −20 °C.

Protein concentration was determined by the Bradford method and BSA (sigma, USA) was used as the standard (Bradford, 1976).

2.4. Assays of protease inhibition

To evaluate the specificity of the recombinant BmSPI, its potency to inhibit subtilisin A (Bacillus sp type VIII, sigma), thrombin (Human plasma, sigma) and a-chymotrypsin (Bovine pancreas, sigma), respectively, was determined using chromogenic