Serpin-5 regulates prophenoloxidase activation and antimicrobial peptide pathways in the silkworm, *Bombyx mori*

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Abstract

The prophenoloxidase (PPO) activation pathway and Toll pathway are two critical insect immune responses against microbial infection. Activation of these pathways is mediated by an extracellular serine protease cascade, which is negatively regulated by serpins. In this study, we found that the mRNA abundance of silkworm serpin-5 (BmSpn-5) increased dramatically in the fat body after bacterial infection. The expression level of antimicrobial peptides (AMPs), gloverin-3, cecropin-D and -E decreased in the silkworm larvae injected with recombinant BmSpn-5 protein. Meanwhile, the inhibition of beads melanization, systemic melanization and PPO activation by BmSpn-5 was also observed. By means of immunoaffinity purification and analysis by mass spectrometry, we identified that the silkworm clip domain serine proteases BmHP6 and SP21 form a complex with BmSpn-5, which suggests that BmHP6 and SP21 are the cognate proteases of BmSpn-5 and are essential in the serine protease cascade that activates the Toll and PPO pathways. Our study provides a comprehensive characterization of BmSpn-5 and sheds light on the multiple pathways leading to PPO activation and their regulation by serpins.

1. Introduction

Insects have evolved efficient innate immune systems to defend themselves against the invasion of pathogens and parasites (Lemaitre and Hoffmann, 2007). The production of antimicrobial peptides (AMPs) and melanization are two key responses in the humoral immunity. In *Drosophila melanogaster*, the mRNA of AMPs is transcribed through Toll or IMD (Immune Deficiency) pathways when the invading bacteria or fungi are recognized as non-self. Specifically, the IMD pathway is activated by the recognition of Gram-negative bacteria, whereas the Toll pathway is activated by the recognition of Gram-positive bacteria and fungi (Imler, 2014). Apart from NK- κ B factor-mediated pathways, melanization is another universal defense mechanism in arthropods that is catalyzed by phenoloxidase (PO), which is proteolytically transformed from zymogen prophenoloxidase (PPO) to its active conformation (Jiang et al., 1998; Lu et al., 2014). The activation of the Toll pathway and PPO is mediated by an extracellular clip domain serine protease cascade (Kambris et al., 2006; Tang et al., 2006; An et al., 2009; An et al., 2013; Veillard et al., 2015; Kanost and Jiang, 2015).

This serine protease cascade is precisely regulated by serine protease inhibitors (serpins, Spns) (Gubb et al., 2010; Kanost, 1999). Existing in all metazoans, the serpin family has a highly variable primary structure but shares a conserved structural similarity. Most members of this family are proteins between 400 and 500 amino acids in length, with a C-terminal exposed reactive center loop (RCL) that connects β -sheet A and C. The RCL is approximately 20 amino acids in length and acts as a

1 pseudo-substrate for its cognate protease. Following binding to the target protease,
2 serpin is cleaved at the scissile bond (P1-P1') located in the RCL and adopts a relaxed
3 conformation, which results in the formation of a covalent complex between the
4 protease and a large part of serpin (Gettins, 2002). During this process, the RCL is
5 inserted into β -sheet A, and dramatic conformational change of the protease causes it
6 to lose its catalytic activity. Serpin genes have been identified systematically in many
7 insect species including *Drosophila melanogaster* (Reichhart, 2005), *Anopheles*
8 *gambia* (Gulley et al., 2013; Suwanchaichinda and Kanost, 2009), *Bombyx mori* (Zou
9 et al., 2009) and *Helicoverpa armigera* (Xiong et al., 2015). In *Drosophila*, Spn43Ac
10 (necrotic, CG1857) and Spn1 (Spn42Dd, CG9456) negatively regulate the Toll
11 signaling pathway (Fullaondo et al., 2011; Levashina et al., 1999). Spn27A
12 (CG11331), Spn28D (CG7219) and Spn77Ba (CG6680) down-regulate the PPO
13 cascade in *Drosophila* (De Gregorio et al., 2002; Ligoxygakis et al., 2002; Tang et al.,
14 2008). In the tobacco hornworm, *Manduca sexta*, Spn-1J, -3, -4, -5, -6, and -7 have
15 been shown to regulate PPO activation and the Toll pathway (An and Kanost, 2010;
16 An et al., 2011; Suwanchaichinda et al., 2013; Tong and Kanost, 2005; Zhu et al.,
17 2003; Zou and Jiang, 2005). In the larval beetle *Tenebrio molitor*, several serpins have
18 been identified as negative regulators of the PPO and Toll pathways (Jiang et al., 2009;
19 Jiang et al., 2011). An ortholog of *M. sexta* Spn-3 in Asian corn borer, *Ostrinia*
20 *furnacalis*, plays roles in both the regulation of PPO activation and antimicrobial
21 peptide production (Chu et al., 2015).

22 In the silkworm, *Bombyx mori*, 34 serpin genes have been identified and their

1 expression patterns in response to microbial infection have been profiled (Zou and
2 Jiang, 2005; Zou et al., 2009). The cDNA of silkworm Spn-2 (also known as
3 antichymotrypsin, sw-Achy), antichymotrypsin II and Spn-9 (also known as
4 antitrypsin, sw-AT) were cloned approximately two decades ago (Narumi et al., 1993;
5 Sasaki, 1991; Takagi et al., 1990). However, there are no functional studies for these
6 serpins. A recent study showed that a silk gland-specific serpin (Spn-16) is a cysteine
7 protease inhibitor, rather than a serine protease inhibitor (Guo et al., 2015). Its
8 physiological function in the silk gland is unclear. *B. mori* Spn-15 is induced
9 by *Beauveria bassiana* and *Micrococcus luteus* and plays a role as a negative
10 regulator in PPO activation and Toll pathways (Liu et al., 2015). In this study, we
11 found that the expression of silkworm serpin-5 (BmSpn-5) in the fat body is
12 remarkably induced by bacterial infection. We used the recombinant BmSpn-5 protein
13 to show that it down-regulates AMPs, gloverin-3, cecropin-D and -E generation and
14 PPO activation. We further revealed that the silkworm clip domain serine proteases,
15 BmHP6 and BmSP21, form complexes with BmSpn-5 using immunoaffinity
16 chromatography and mass spectrometry analysis. This suggests that BmHP6 and
17 BmSP21 are the target proteases and participate in the serine protease cascade that
18 leads to activation of the Toll and PPO pathways.

19 **2. Materials and Methods**

20 *2.1. Silkworm rearing and infection with bacteria*

21 The silkworm *B. mori* (*Nistari* strain) was reared on mulberry leaves at 27 °C in
22 70% RH and a photoperiod of 13:11 (light:dark). *Micrococcus luteus* and *Yersinia*

1 *pseudotuberculosis* serotype O:3 (YPIII) were cultured at 37 °C in LB medium
2 overnight. The cells were collected by centrifugation at 8000 × g. The pellet was
3 resuspended in 1 ml sterilized 0.85% NaCl, then 40 ml 70% isopropyl alcohol was
4 added and incubated for 1 hour at room temperature with rotation to kill the cells.
5 After three rounds of washing with sterilized 0.85% NaCl, the isopropanol-killed
6 bacteria were resuspended in sterilized 0.85% NaCl to OD_{600nm} of 1.0. 50 µl of the
7 bacterial preparations were injected into the hemocoel of day-3 fifth-instar silkworm.

8 2.2. Analysis of *BmSpn-5* expression by quantitative real-time PCR.

9 The day-3 fifth-instar larvae were injected with either sterilized 0.85% NaCl (as
10 control), isopropanol-killed *M. luteus* or YPIII cells. At 6, 24 and 48 hours post
11 infection (hpi), fat body from 5-8 larvae were collected in 500 µl TriPure Reagent
12 (Roche, USA) and stored at -80 °C. Hemolymph from at least 15 larvae was pooled
13 and spun at 500 × g for 10 min at 4 °C to collect hemocytes. After discarding the
14 supernatant, the hemocytes were mixed with TriPure Reagent and stored at -80 °C.
15 Total RNA was extracted from the stored fat body and hemocytes samples following
16 the standard protocol provided by the producer. 30 µg total RNA was further purified
17 with Direct-zol™ RNA MiniPrep (Zymo Research, USA), and first strand cDNA was
18 synthesized by Transcriptor First Strand cDNA Synthesis Kit (Roche, USA). Using
19 the prepared cDNA as a template, quantitative real-time PCR (qPCR) was carried out
20 by 2 × FastStart Essential DNA Green Master (Roche), with 40 thermal cycles (94 °C,
21 15 s; 55 °C, 20 s; 72 °C, 20 s) after initial denaturation at 95 °C for 10 min. A melt
22 curve (65 – 95 °C) was determined to confirm the amplification of specific PCR

1 product (Bio-Rad IQ5). The *Bombyx* housekeeping gene *IF4A* (DQ443290.1) was
2 used as an internal control to normalize the transcript level of *BmSpn-5* (Wu et al.,
3 2010). The standard curve of each gene was measured to determine the amplification
4 efficiency and r^2 value. The relative quantitative method ($2^{-\Delta\Delta C_t}$) was applied to
5 determine the expression level of *BmSpn-5* (Schmittgen and Livak, 2008). Primers
6 used for quantitative real-time PCR are listed in Table 1.

7 2.3. Expression and purification of recombinant *BmSpn-5*

8 5' RACE-ready cDNA was synthesized from the total RNA from the midgut of
9 the fifth instar silkworm using SMARTer RACE cDNA amplification kit (Clontech,
10 USA) according to the provided manual. The cDNA fragment encoding *BmSpn-5*
11 (GenBank number: AY566165.1) was amplified with gene specific primers, in which
12 a *Bam*HI and an *Xho*I restriction site were introduced (Table 1). The amplified
13 product was cloned in the pET32a expression vector (Novagen, USA). The expression
14 of *BmSpn-5* in the *E. coli* BL21 (DE3) strain was induced by 0.5 mM
15 isopropyl-beta-D-thiogalactopyranoside (IPTG). The collected cells were resuspended
16 in phosphate buffered saline (PBS) and homogenized by sonication (Sonics
17 Vibra-Cell, 130 w, 30% power). *BmSpn-5* was purified from the soluble fraction first
18 using a Ni^{2+} -NTA column by a stepwise elution in PBS containing 25 mM, 50 mM,
19 150 mM, and 500 mM imidazole. The fractions (50-150 mM imidazole) containing
20 recombinant *BmSpn-5* were pooled and dialyzed in Tris-HCl buffer (20 mM Tris, 50
21 mM NaCl, 2 mM $CaCl_2$, pH 7.4), followed by digestion with recombinant
22 enterokinase (rEK) (Amoytop Biotech, China) for 5 hours. The digested protein was

1 purified on Ni²⁺-NTA column again. Due to the rEK digestion, the Trx tag, His tag
2 and S tag were cleaved from BmSpn-5, resulting in the loss of BmSpn-5 affinity for
3 the Ni²⁺-NTA column. After dialysis with 50 mM Tris-HCl (pH 8.2), recombinant
4 BmSpn-5 was further purified by Q-Sepharose Fast Flow column in 50 mM Tris-HCl
5 (pH 8.2) with a stepwise elution containing 50 mM, 150 mM, 300 mM, and 500 mM
6 NaCl. BmSpn5 was eluted in the fraction containing 150 mM and 300 mM NaCl, and
7 then desalted by dialysis. Protein concentration was determined using the BCA
8 Protein Assay Kit (Beyotime, China). Purified recombinant BmSpn-5 was verified by
9 peptide mass fingerprinting (Beijing Protein Innovation). A rabbit polyclonal
10 antiserum was prepared against the recombinant BmSpn-5 (Cwbio, China).

11 *2.4. SDS-PAGE and immunoblotting*

12 Recombinant BmSpn-5 was analyzed by 12% SDS-PAGE and stained with
13 Coomassie brilliant blue dye. For the western blot, separated proteins were
14 electrotransferred onto a PVDF membrane (Millipore). The membrane was
15 subsequently blocked with 5% skim milk powder (Merck) in TBST (0.05% Tween-20,
16 150 mM NaCl, 50 mM Tris). Anti-His rabbit polyclonal antibody (1:5000 dilution)
17 (Santa Cruz Biotechnology) and anti-BmSpn-5 rabbit polyclonal antibody (1:10000
18 dilution) were used as primary antibodies, and goat-anti-rabbit IgG (1:10000 dilution)
19 (Santa Cruz Biotechnology) was used as the secondary antibody. Western blotting
20 detection kit (Advasta) was used for detection and a chemiluminescence imaging
21 system was used to document the images (Clinx Science Instruments Co., Ltd).

22 *2.5. Melanization assay of Sepharose beads in the silkworm hemolymph*

1 Positively charged QAE Sepharose Fast Flow (QFF) chromatography beads
2 (50-150 μm in diameter, Sigma) were used for analysis of encapsulation in silkworm
3 hemolymph. QFF beads were stained fully with 0.1% Congo red for 2 hours and
4 washed three times with 50 mM Tris-HCl buffer (pH 8.2). The washed beads were
5 resuspended in 1 ml of serpin-5 (300 $\mu\text{g}/\text{ml}$, in 50 mM Tris-HCl, pH8.2) to
6 approximately 100 beads per 50 μl and incubated for 1 hour at room temperature with
7 gentle rotation. The serpin-5 coated beads were washed three times with Tris-HCl
8 buffer and resuspended in Tris-HCl buffer. Fifteen day 3 fifth-instar larvae silkworm
9 larvae were anesthetized on ice and 50 μl of the bead suspension was injected into the
10 larval hemocoel. The larvae were dissected 4 hours later and the beads were recovered
11 and observed under microscope. Bovine serum albumin (BSA) coated beads were
12 injected as a control.

13 2.6. *Prophenoloxidase activation and phenoloxidase activity assay*

14 Day-3 fifth-instar larvae were injected with 50 μl BSA (150 $\mu\text{g}/\text{ml}$ in 50 mM
15 Tris-HCl, pH 8.0) or 50 μl recombinant BmSpn-5 (150 $\mu\text{g}/\text{ml}$ in 50 mM Tris-HCl, pH
16 8.0). After 30 min, hemolymph was collected and centrifuged immediately at 16000 $\times$
17 g for 30 seconds at 4 $^{\circ}\text{C}$ to remove hemocytes. 5 μl of plasma sample was added to
18 the wells of a 96 well plate, and 5 μl of 1 mg/ml pathogen-associated molecular
19 patterns (PAMPs), polysaccharides (LPS) from *Porphyromonas gingivalis*, curdlan
20 from *Alcaligenes faecalis*, or peptidoglycan (PG) from *E. coli* or *M. luteus* and 100 μl
21 of 2 mM dopamine were added and then mixed immediately. The absorption at 490
22 nm was recorded every 30 seconds for 15 min on a microplate reader (Tecan), where

1 the maximum slope was determined. 60 μ l of the two groups freshly prepared plasma
2 were added simultaneously to each 1.5 ml Eppendorf tubes that contained 10 μ l of 1
3 mg/ml elicitor and incubated at room temperature. At 1 min, 5 min and 25 min, 20 μ l
4 of each sample was taken and mixed with 20 μ l of 4 $\times$ SDS loading buffer and boiled
5 immediately at 100 °C for 5 min. After centrifugation at 5000 $\times$ g for 3 min, the
6 samples were analyzed by 8% SDS-PAGE and western blot with a mixture of
7 anti-PPO1 and anti-PPO2 (1:5000 dilution) (gifts from Dr. Michael Strand) as the
8 primary antibodies.

9 2.7. Immunoaffinity purification of *BmSpn-5*-protease complexes

10 Anti-BmSpn-5 covalent coupled Protein A-agarose was prepared according to a
11 previous study (Jedamzik and Eckmann, 2009) that used dimethyl pimelimidate
12 (DMP) (Sigma-Aldrich) as the linker. 2-3 ml of hemolymph from day 3 fifth instar
13 naïve larvae or larvae that were injected with dead *M. luteus* were collected into a
14 tube and 10 μ l of 1% phenylthiourea (PTU) was added. Hemocytes were then
15 removed by centrifugation at 500 $\times$ g for 10 min at 4 °C. PMSF (final concentration
16 of 4 mM) and protease inhibitor cocktail (Thermo) were added to prevent protease
17 activity. For another hemolymph sample of *M. luteus* injected larvae, *M. luteus*-PG
18 was added to a final concentration of 1 mg/ml, followed by incubation at room
19 temperature for 20 min. *M. luteus*-PG was then removed by centrifugation at 10000 $\times$
20 g for 5 min at 4 °C. PMSF and protease inhibitor cocktail were also added in the same
21 amount. All three groups of samples were subsequently incubated with 50 μ l of
22 Protein A-agarose beads for 5 hours at 4 °C to remove the non-specific absorption.

1 After centrifugation at $12000 \times g$ for 1 min, the supernatants were added to 50 μ l of
2 anti-BmSpn-5 coupled Protein A-agarose beads and incubated overnight at 4 °C.
3 BmSpn-5-protease complexes and BmSpn-5 were then washed and eluted as
4 described in the Protein A Immunoprecipitation Kit (Roche). Eluted proteins were
5 then separated by 4%-12% NuPAGE BIS-Tris Gels with MOPS SDS running buffer
6 (life technology), followed by modified Blum silver staining (Mortz et al., 2001),
7 which is compatible with mass spectrometry analysis. Anti-BmSpn-5 was used to
8 detect BmSpn-5-protease complexes in western blots. Protein bands on the silver
9 stained gel were cut in accordance to bands on the western blot, and then digested
10 with trypsin (Promega). The resulting peptide mixture was pre-separated by the
11 Eksigent nanoLC-Ultra™ 2D System (AB SCIEX) equipped with a C18 nanoLC trap
12 column (100 μ m $\times$ 3 cm, 3 μ m, 150 Å). Then, an analytical ChromXP C18 column
13 (75 μ m x 15 cm, 3 μ m, 120 Å) with a spray tip was used to separate the peptide
14 mixture. Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX,
15 USA) fitted with a Nanospray III source (AB SCIEX) and a pulled quartz tip as the
16 emitter (New Objectives). Tandem mass spectra were searched with MASCOT V2.3
17 search engine (Matrix Science Ltd.) and identifications were confirmed by manual
18 evaluation.

19 2.8. Hemolymph antibacterial activity assay

20 Day 3 fifth instar larvae were anesthetized on ice for 15 min, followed by injection
21 with 30 μ g BSA or 30 μ g recombinant BmSpn-5 in 50 μ l PBS. After 30 min of
22 incubation in a rearing incubator, the larvae were anesthetized on ice for another 15

1 min. Then, the two groups of silkworm were then immune challenged by
2 isopropanol-killed *M. luteus* and YPIII. Additionally, 50 µl 0.85% NaCl was injected
3 into another BSA pre-injection group as control. Fat body from at least 5 silkworm
4 larvae was collected 24 h after the first injection. The expression of AMPs was
5 subsequently analyzed by quantitative real-time PCR (Table 1). In 24 h after the first
6 injection, plasma samples were collected and prepared as described before⁷. The
7 antimicrobial activity assay of plasma was performed by measuring zones of growth
8 inhibition in a thin layer of agar plates, as described previously (Hoffmann et al.,
9 1981). *M. luteus* and YPIII were used as the detective strains. Moreover, 5-µl plasma
10 samples were treated with 5 µl 2 × SDS loading buffer at 100 °C for 5 min and
11 separated by electrophoresis on a 16% tricine-SDS-PAGE (Schagger, 2006) and
12 stained with Coomassie brilliant blue. The bands were excised and digested with
13 In-Gel Tryptic Digestion Kit (Thermo). LC-MS Analysis was completed using a
14 LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific). Liquid
15 chromatography separation was carried out on a BioBasic-18 Column (150 mm
16 ×0.075 mm, Thermo Fisher). Peptides were eluted with a 60-min linear gradient at a
17 flow rate of 1 µl/min from 5 to 35 % acetonitrile containing 0.1% formic acid. The
18 mass spectrometer worked in the positive nano-electrospray ionization (NSI) mode.
19 The spray voltage was set at 2.5 kV and the ion transfer capillary temperature was
20 275 °C. Data were searched in the Swiss-Port Protein Sequence Database using
21 Proteome Discoverer (version 1.1, Thermo Fisher Scientific).

22 2.9. Statistical analysis

Antibacterial assay, PO activity assay and qPCR experiments were repeated independently at least three times. Graphpad Prism 5.0 was use to plot the data. Student's *t*-test and Bonferroni's test were used to analyze the statistical significance. All data are presented as the means $\pm$ SD.

3. Results

3.1. Sequence and phylogenetic analysis of *BmSpn-5*

BmSpn-5 cDNA (GenBank accession number: AY566165) encodes a polypeptide of 395 amino acid residues, with a predicted signal peptide that consists of the first 16 residues. The calculated molecular weight and isoelectric point of the mature protein are 42.63 kDa and 6.02, respectively. *BmSpn-5* is orthologous to *M. sexta* serpin-5, with 79% identity in amino acid sequence. Phylogenetic analysis also revealed that *BmSpn-5* is in the same clade as *Manduca* Spn-4, -5, and -7 (Fig. 1A). The sequence alignment of the RCL regions indicated the same predicted P1-P1' (Arg-Ile) residues of these four serpins (Fig. 1B). The high similarity between these serpins suggests that they might share similar regulatory functions.

3.2. Bacterial infection induces *BmSpn-5* expression in the fat body of larval silkworm

Fat body and hemocytes are the major tissues responsible for immune responses in insects (Kounatidis and Ligoxygakis, 2012; Lavine and Strand, 2002). We therefore examined the expression of *BmSpn-5* in hemocytes after bacterial infection. Quantitative real-time PCR results showed that the *BmSpn-5* transcriptional level

1 significantly increased in the fat body during 48 hours post infection (hpi), and peaked
2 approximately 10-20-fold at 6 hpi (Fig. 2A). In the hemocytes, mRNA abundance of
3 BmSpn-5 was up-regulated at 48 h after bacterial infection (Fig. 2B).

4 3.3. *Production of recombinant BmSpn-5*

5 To investigate its physiological function, we expressed and purified recombinant
6 BmSpn-5 from *E. coli*. BmSpn-5 was expressed as a fusion protein with a thioredoxin
7 (Trx) and a hexahistidine tag in the N-terminus to facilitate its correct folding and
8 purification. The fusion protein was purified by nickel-affinity chromatography from
9 the soluble fraction. The Trx-hexahistidine tag was cut from BmSpn-5 after digestion
10 with enterokinase, and separated from BmSpn-5 due to its ability to bind to the nickel
11 column. The purified recombinant BmSpn-5 was verified by mass spectrometry (data
12 not shown). Figure 3 illustrates the induction and purification of BmSpn-5 as shown
13 in the SDS-PAGE stained with Coomassie blue (Fig. 3A) and immunoblots using
14 anti-His antibody (Fig. 3B) and anti-BmSpn-5 antibody (Fig. 3C).

15 3.4. *BmSpn-5 inhibits bead melanization in the silkworm hemolymph*

16 When foreign intruders, such as beads and parasitic wasp eggs, are introduced
17 into the hemocoel of host insects, a melanotic capsule will form around the beads or
18 eggs (Michel et al., 2006; Nappi et al., 2005). Similarly, the sepharose beads injected
19 into the larval silkworm hemocoel were melanized by the hemolymph (as seen under
20 stereoscopic microscope, Fig. 4A) and were also surrounded by a layer of hemocytes
21 (as seen under phase contrast microscope, Fig. 4A). At four hours after injection,
22 approximately 25 percent of the BSA-coated beads were melanized, whereas only

1 approximately 5 percent of the BmSpn-5-coated beads were melanized (Fig. 4B),
2 suggesting that BmSpn-5 inhibits hemolymph melanization in the silkworm larvae.

3 3.5. *Bm-serpin5 inhibits systemic melanization in the larval silkworm*

4 When we injected the larval silkworm with dead bacterial *YP* ~~III~~ells, we found that
5 the treated animals were inert and the body color was darkened twelve hours later.
6 The plasma sample collected from the larvae was dark and there were plenty of
7 nodules in the hemocoel. On the contrary, if we injected the larvae with recombinant
8 BmSpn-5 before the injection of bacterial cells, these larvae were still active and their
9 plasma remained a yellow color, and there were no apparent clots found in the
10 hemocoel (Fig. 5). These observations indicate that BmSpn-5 is able to block
11 systemic melanization triggered by dead bacterial cells.

12 3.6. *BmSpn-5 inhibits PPO activation.*

13 Recognition of pathogen-associated molecular patterns (PAMPs), such as LPS,
14 PG from bacteria, and curdlan from fungi, by pattern recognition proteins (PRPs)
15 initiates the sequential activation of a serine protease cascade in the hemolymph and
16 consequently results in the activation of melanization (Jiang et al., 2010). This process
17 is under the regulation of serpins (Gubb et al., 2010; Kanost, 1999). We postulate that
18 BmSpn-5 might regulate PPO activation in silkworm. The larval silkworm was
19 injected with recombinant BmSpn-5, and shortly afterward was treated with distinct
20 types of PAMPs to trigger the immune response *in vitro*. Apart from the activation by
21 *M. luteus*, pre-injection of BmSpn-5 also inhibited the PO activity that resulted from
22 the activation by PG from *E. coli*, LPS, and curdlan (Fig. 6A). A high molecular mass

1 complex formation is required for maintaining PO activity in the silkworm (Clark and
2 Strand, 2013). Therefore, we inspected the formation of the complex in the process of
3 activation (Fig. 6B). When the silkworm larvae were injected with bovine serum
4 albumin (BSA) and the plasma was incubated with LPS, PG, and curdlan, we
5 observed cleavage of PPO and the formation of the PO complex. In contrast, in the
6 presence of BmSpn-5, both the cleavage of PPO and the formation of the PO complex
7 were partially inhibited (in the activation by curdlan and PG from *M. luteus*) or
8 completely abolished (in the activation by LPS and PG from *E. coli*). These results
9 demonstrate that BmSpn-5 down-regulates the PPO pathway when it is specifically
10 activated by PG and LPS from Gram-negative bacteria and curdlan from fungi.

11 3.7. *BmSpn-5 inhibits AMP generation induced by bacteria*

12 The transcription and translation of AMPs in the fat body was elicited by
13 microbial infection and finally released into the hemolymph (Imler, 2014). We
14 challenged larval silkworms with bacteria *M. luteus* and *YP111*. Remarkable
15 antibacterial activity was detected in the hemolymph 24 hours after bacterial infection.
16 However, pre-injection of recombinant BmSpn-5 into the hemocoel decreased the
17 antibacterial activity induced by both *M. luteus* and *YP111*, in a dosage-dependent
18 manner when the silkworms were treated with *M. luteus* (Fig. 7A). Next we examined
19 the composition in the small molecule portion of challenged hemolymph in a
20 tricine-SDS-PAGE. It appeared that proteins induced by bacterial infection were
21 located at approximately 16 kDa (band 1 in Fig. 7B) and 3.5 kDa (band 2), whereas
22 pre-injection of BmSpn-5 inhibited their induction in the hemolymph. LC/MS-MS

1 analysis identified that band 1 is gloverin and that band 2 corresponds to cecropin and
2 moricin (Fig. 7C). Our semi-quantitative PCR showed that *gloverin-3*, *cecropin-D* and
3 *cecropin-E* are strongly induced in the fat body by *M. luteus* and *YPH* infection and
4 are down-regulated by recombinant BmSpn-5 during *M. luteus* infection (data not
5 presented). Quantitative real-time PCR confirmed that *gloverin-3*, *cecropin-D* and
6 *cecropin-E* are negatively regulated by BmSpn-5 after *M. luteus* challenge;
7 *cecropin-E* is also negatively regulated after *YPH* challenge (Fig. 7D). These results
8 indicate that BmSpn-5 plays a role as an inhibitor in the immune response against *M.*
9 *luteus*.

10 3.8. Silkworm SP21 and BmHP6 are candidates for the BmSpn-5 target protease.

11 Our results show that BmSpn-5 functions as an inhibitor to both PPO and
12 AMP-producing pathways. To further identify its target protease, we attempted to
13 capture BmSpn-5-protease complexes. In the unchallenged plasma, we detected a ~50
14 kDa band corresponding to BmSpn-5 (lane 1 in Fig. 8A). In the challenged plasma,
15 the intensity of the ~50 kDa band increased, and an extra lower band was observed
16 (lane 2 in Fig. 8A). This indicates that BmSpn-5 is induced and processed by immune
17 challenge in the plasma. When the challenged plasma was activated by PG, we
18 detected two extra bands approximately 70 kDa, the expected position of
19 serpin-protease complex (lane 3 in Fig. 8A). LC/MS-MS analysis revealed that two
20 clip domain serine proteases, BmHP6 (AK384444, silkworm full-length cDNA
21 database KAIKO_cDNA) and BmSP21 (NP_001243984), are identified in complex 1
22 and 2, respectively (Fig. 8A and 8B). The immunoblotting and mass spectrometry

analysis showed that BmHP6 and BmSP21 could form complexes with BmSpn-5, suggesting that these two serine proteases are the target proteases of BmSpn-5.

4. Discussion

Serpins are involved in the regulation of multiple physiological and immune signaling pathways, such as hemolymph clotting, PPO activation, and the proteolytic activation of cytokines (Gubb et al., 2010; Kanost, 1999). In this study, we demonstrate that BmSpn-5 in the silkworm negatively regulates the production of AMPs and the PPO activation pathways. The activation of AMP synthesis and PPO pathways in insects depends on an extracellular clip domain serine protease cascade, which is under strict regulation by serpins. BmSpn-5 forms covalent complexes with either BmSP21 or BmHP6, which implies that BmSpn-5 may inhibit the cleavage and activation of the downstream proteases involved in these two pathways.

The inhibitory selectivity of serpins is largely determined by the residues adjacent to the scissile bond. We compared the predicted cleavage sites of BmSpn-5 and serine proteases identified as the components of the cascade leading to PPO and Toll pathways in *Bombyx*, *Manduca*, *Tenebrio* and *Drosophila* (Fig. 8C). *Bombyx* BAEEase and PPAE have similar activation sites (Arg-Ile and Lys-Ile, respectively) to BmSpn-5 (Arg-Ile). This suggests that BAEEase, together with PPAE, may be cleaved and activated by BmSP21 or BmHP6. The zymogen of *Bombyx* BAEEase was activated in the silkworm hemolymph when PG or β -1,3-glucan was present. Its homolog in *Drosophila*, spätzle-processing enzyme (SPE), is required for Toll activation and host defense (Jang et al., 2006). The activation site of *Bombyx*

1 BAEase is similar to that of *Drosophila* SPE (Jang et al., 2006), *Tenebrio* SPE (Kim
2 et al., 2008) and *Manduca* HP8, which serves as the SPE (An et al., 2009; An and
3 Kanost, 2010; An et al., 2011) in the Toll pathway. Together with our evidence that
4 BmSpn-5 inhibits AMP generation, we hypothesize that BAEase might play a
5 critical role similar to the SPE and could be activated by BmSP21 or BmHP6 in the
6 silkworm.

7 In *Manduca*, HP8 is activated by HP6, which is the target protease of serpin-5.
8 *Manduca* HP6 also cleaves PAP1, which activates PPO (An and Kanost, 2010).
9 Comparison of the sequences of *Manduca* HP6 and BmHP6 shows that they share 66%
10 identity of amino acid residues and have similar activation sites, with His-Ile in HP6
11 and Tyr-Ile in BmHP6 (Fig. 8C). *Tenebrio* SPE is capable of cleaving PPO and
12 pro-spätzle, which activates melanization and the Toll pathways. *Tenebrio* pro-SPE is
13 activated by SAE (SPE-activating enzyme), which is negatively regulated by
14 serpin-55 (Jiang et al., 2011). BmSP21 shares 56% identity of amino acid sequence
15 with *Manduca* HP21 and 38% identity with *Tenebrio* SAE. All three of these
16 proteases share similar activation sites (Leu-Ile), which suggests that they could be
17 cleaved by the same protease (Fig. 8C). *Manduca* HP21 cleaves pro-PAP2 and
18 pro-PAP3, resulting in the activation of PPO (Gorman et al., 2007; Wang and Jiang,
19 2007). Similarly, the *Bombyx* PPO-activating enzyme (PPAE, KAIKO_cDNA:
20 AK383065) has two clip domains at its amino terminus and is predicted to be
21 activated at Lys¹⁵²-Ile¹⁵³ (Sato et al., 1999). PPAE was identified as the ortholog of
22 *Manduca* PAP3 (Tokura et al., 2014). We also identified BmPAP2 (AK386026) as the

1 ortholog of *Manduca* PAP2 by using TBALSTN, the silkworm full-length cDNA
2 database. Therefore, BmSP21 may also cleave and activate pro-PPAE and
3 pro-BmPAP2, in addition to pro-BAEEase.

4 A recently identified ortholog of *Manduca* PAP1 in *Bombyx* (SilkDB:
5 BGIBMGA010546) suggests that there is more than one protease cascade leading to
6 the activation of PPO in the silkworm (Tokura et al., 2014), which resembles the PPO
7 activation in *Manduca* (Tong and Kanost, 2005; An and Kanost, 2010). Another
8 interesting observation in our study is that inactive *M. luteus* induced up-regulation of
9 gloverin-3, cecropin D and cecropin E and this up-regulation was inhibited by
10 BmSpn-5 (Fig. 7D). However, the activation of PPO by PG from *M. luteus* was not
11 inhibited efficiently by BmSpn-5 (Fig. 6). This suggests that the protease cascade
12 leading to activation of PPO by *M. luteus* may bypass BmSP21 and BmHP6.
13 Additionally, the fact that BmSP21 was present in the melanotic nodules induced by *E.*
14 *coli*, but not in those induced by *M. luteus* or *S. cerevisiae* (Tokura et al., 2014),
15 implies the possible existence of multiple PPO activation pathways in the silkworm.

16 Taken together, we have shown that BmSpn-5 down-regulates the Toll and PPO
17 pathways by targeting BmHP6 and BmSP21 in the silkworm. Our study provides
18 clues for further dissection of the silkworm Toll and PPO activation signaling.

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Figure legend

Figure 1 Phylogenetic analysis (A) and RCL region sequences comparison (B) of BmSpn-5 and serpins of *Manduca Sexta* (Ms) and *Drosophila melanogaster* (Dm). The selected serpin protein sequences were aligned using ClustalX. (A) The phylogram was built by neighbor-joining method. The numbers on the branches represent bootstrap values. (B) Alignment of the RCL sequences containing the scissile bond and activation sites. The P1-P1' scissile bond is indicated by an arrow.

Dm_spn28Dc: NP_609172; Dm_spn77Ba: NP_730512.2; Dm_spn42Dd: ACZ94347; Dm_spn27A: AAF52420; Ms_spn1J: AAC47340; Ms_spn3: AAO21505; Ms_spn4: AAS68503; Ms_spn5: AAS68507; Ms_spn6: AAV91026; Ms_spn7: ADM86478.

Figure 2 mRNA abundance of *BmSpn-5* in fat body and hemocytes after bacterial injection. Fat body (A) and hemocyte (B) samples were collected at 12 hpi to analyze the transcription levels of *HaPGRP-A* using qPCR. Error bars show means $\pm$ SD ($n = 3$). Significant differences between the bacterial infected samples and the NaCl injected samples are indicated with * at $P < 0.05$, ** at $P < 0.01$ and *** at $P < 0.001$, respectively.

Figure 3 Expression and purification of recombinant BmSpn-5. (A) Coomassie brilliant blue stained SDS-PAGE. (B) Western blot probed with anti-His antibody. (C) Western blot probed with anti-BmSpn-5 antibody. M, protein molecular mass markers; lane 1, total proteins from uninduced *E. coli* cells; lane 2, total proteins from induced *E. coli* cells; lane 3, soluble proteins from induced *E. coli* cells; lane 4, purified fusion

protein before enterokinase digestion; lane 5, purified BmSpn-5.

Figure 4 BmSpn-5 inhibits beads melanization in the hemolymph of larval silkworm. (A) Sepharose beads coated with BSA or recombinant BmSpn-5 were injected into the hemocoel of silkworm larvae. The beads were recovered from dissected larvae and observed under phase contrast microscope (PCM) and stereoscopic microscope (SM) 4 hours post injection. (B) The percentage of melanized beads in the hemolymph. Error bars show mean $\pm$ SEM. Significant differences between the treatment and the control are analyzed by the student *t* test.

Figure 5 BmSpn-5 inhibits systemic melanization. These photographs show whole animals, plasma, and tissues after pre-injection of 30 μ g of BSA (left panel) or BmSpn-5 (right panel) and following approximately 2×10^8 dead *YP111* bacterial cells injection. The samples were collected and observed twelve hours after injection of bacterial cells.

Figure 6 BmSpn-5 down-regulates PPO activation. (A) The PO activity of the hemolymph is measured from larvae pre-injected with BmSpn-5 or BSA and then activated with pathogen-associated molecular patterns (PAMPs, including LPS, curdlan and PG). (B) Western blots showing PO complex formation in the hemolymph pre-injected with BmSpn-5 or BSA and activated with LPS, curdlan, or PG. The arrowheads in C indicate the PO complex in the plasma; white and black arrows indicate PPO and PO, respectively.

Figure 7 The production of AMPs was down-regulated by BmSpn-5. (A) Antibacterial activity of the hemolymph determined by inhibition zone method. (B)

1 Plasma proteins separated on a Tricine-SDS-PAGE and visualized by Coomassie
2 brilliant blue stain. Note only the lower part of the gel was shown. The boxes indicate
3 the bands of interest. (C) The mass spectrometry results of the protein bands excised
4 from the gel in (B). (D) Antimicrobial peptide mRNA abundance in fat body
5 determined by qPCR. Error bars show mean $\pm$ SD ($n = 5$). Significant differences
6 between the indicated groups are marked with * at $P < 0.05$, ** at $P < 0.01$ and *** at
7 $P < 0.001$, respectively.

8 **Figure 8 Capture and identification of the BmSpn-5-protease complexes. (A)**

9 Immunoaffinity purification of the BmSpn-5-protease complexes. Left, silver stained
10 SDS-PAGE; Right, western blot probed with anti-BmSpn-5 antibody. Lane 1,
11 unchallenged plasma; lane 2, *M. luteus*-challenged plasma; lane 3, *M.*
12 *luteus*-challenged plasma activated with PG. (B) Mass and peptides matched to
13 BmHP6 and BmSP21 in the complex 1 and complex 2, respectively. (C) Comparison
14 of activation site sequences between BmSpn-5 and proteases from *Bombyx*,
15 *Drosophila*, *Manduca* and *Tenebrio*. PAP, Prophenoloxidase activating protease; SPE,
16 Spätzle-processing enzyme; SAE, SPE-activating enzyme. The P1-P1' scissile bond is
17 indicated by an arrow.

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B BmHP6

Mr (expt)	Mr (calc)	Score	Expect	Unique	Peptide
934.5228	934.5236	38	0.008	✓	R.LVGVTSFGR.G
1213.5178	1213.5186	35	0.005	✓	K.CSESYLENWR.K
1214.5166	1214.5026	20	0.017	✓	K.CSESYLENWR.K
1248.5924	1248.5921	49	4.50E-05	✓	R.GCGSPVPGVYTR.L
1673.7676	1673.7679	112	9.90E-10	✓	K.GISDSQICAGDPEGIR.D
1706.7148	1706.7141	108	1.70E-09	✓	R.DTCQGDGGPLQMWR.D

BmSP21

Mr (expt)	Mr (calc)	Score	Expect	Unique	Peptide
1024.5704	1024.5705	41	0.0013	✓	R.YVYIGALAR.H
1261.605	1261.6051	76	2.60E-06	✓	R.ASATGWGLTENR.G
1293.5926	1293.5949	80	1.30E-06	✓	R.ASATGWGLTENR.G
1333.6854	1333.6877	69	5.80E-06	✓	K.YNDIALLELDR.Q
1334.6698	1334.6718	61	9.90E-05	✓	K.YNDIALLELDR.Q
1424.673	1424.6847	9	1.2	✓	K.GNLLGVCTNIRK.C
1474.669	1474.6722	109	6.10E-10	✓	R.DTCQGDGGPIQIK.S
2210.0977	2210.0451	12	0.45	✓	R.ASATGWGLTENRGSTSDVLQK.V

C

	P1 P1'
Bombyx Spn5	L-I-D-R-I-S-A-P
Bombyx BAEase	N-N-D-R-I-F-G-G
Bombyx PAP1	Q-G-D-R-I-Y-G-G
Bombyx AK386026 (PAP2)	V-G-N-K-I-V-G-G
Bombyx PPAAE (PAP3)	V-G-D-K-I-V-G-G
Manduca PAP1	N-G-D-R-I-Y-G-G
Manduca PAP2	F-D-N-K-I-L-G-G
Manduca PAP3	V-G-N-K-I-I-G-G
Manduca HP8	N-N-D-R-I-V-G-G
Drosophila SPE	F-A-D-R-I-F-G-G
Tenebrio SPE	T-Q-N-R-I-Y-G-G
Tenebrio SAE	I-I-K-I-I-V-G-G
Bombyx SP21	A-D-E-L-I-I-G-G
Manduca HP21	A-D-D-L-I-I-G-G
Manduca HP6	L-D-L-H-I-L-G-G
Bombyx HP6	L-D-L-Y-I-L-G-G

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1 Table 1 primers used in gene cloning and quantitative real-time PCR

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Primer	Sequence (5' - 3')
<i>BmSpn-5 gene cloning</i>	
Forward:	GCGGATCCGATGTCGATTTTCAGCGAGA
Reverse:	GCTCGAGTCAATAGACAGTCGGTTTTGAA
<i>BmSpn-5 quantitative RT- PCR</i>	
Forward:	GCACCCGAGAACGAAGAT
Reverse:	GGCTAAGTCTGGCTGGAATA
<i>BmIF4A quantitative RT- PCR</i>	
Forward:	TCTGGCATCATACCTTCTACAA
Reverse:	TCTGTGTCATCTTTTCCCTGTT
<i>BmGlov3 quantitative RT-PCR</i>	
Forward:	GACACGAGAATGGGAGGAG
Reverse:	AAGACCCTGGTGCCGTAA
<i>BmCecD quantitative RT-PCR</i>	
Forward:	CTCCCGGCAACTTCTTCA
Reverse:	CGAACCCTCTGACCCATT
<i>BmCecE quantitative RT-PCR</i>	
Forward:	ACTGTTCGACATCGCCTCT
Reverse:	CGAATGTTCTGACCCACC

3 Underline showed the *Bam*HI and *Xho*I restriction enzyme sites.

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