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***Manduca sexta* serpin-12 controls the prophenoloxidase activation system in larval hemolymph**

Fan Yang¹, Yang Wang¹, Niranji Sumathipala¹, Xiaolong Cao¹, Michael R. Kanost², and Haobo Jiang¹

¹Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK, 74078, USA

²Department of Biochemistry and Molecular Biophysics, Kansas State University, Manhattan, KS, 66506, USA

Abstract

Insect prophenoloxidase activation is coordinated by a serine protease network, which is regulated by serine protease inhibitors of the serpin superfamily. The enzyme system also leads to proteolytic processing of a Spätzle precursor. Binding of Spätzle to a Toll receptor turns on a signaling pathway to induce the synthesis of defense proteins. Previous studies of the tobacco hornworm *Manduca sexta* have revealed key members of the protease cascade, which generates phenoloxidase for melanogenesis and Spätzle to induce immunity-related genes. Here we provide evidence that *M. sexta* serpin-12 regulates hemolymph protease-14 (HP14), an initiating protease of the cascade. This inhibitor, unlike the other serpins characterized in *M. sexta*, has an amino-terminal extension rich in hydrophilic residues and an unusual P1 residue (Leu⁴²⁹) right before the scissile bond cleaved by a target protease. Serpins with similarities to serpin-12, including *Drosophila* Necrotic, were identified in a wide range of insects including flies, moths, wasps, beetles, and two hemimetabolous species. The serpin-12 mRNA is present at low, constitutive levels in larval fat body and hemocytes and becomes more abundant after an immune challenge. We produced the serpin-12 core domain (serpin-12 N) in insect cells and in *Escherichia coli* and demonstrated its inhibition of human cathepsin G, bovine α -chymotrypsin, and porcine pancreatic elastase. MALDI-TOF analysis of the reaction mixtures confirmed the predicted P1 residue of Leu⁴²⁹. Supplementation of larval plasma samples with the serpin-12 N decreased prophenoloxidase activation elicited by microbial cells and reduced the proteolytic activation of the protease precursors of HP6, HP8, PAPs, and other serine protease-related proteins. After incubation of plasma stimulated with peptidoglycan, a 72 kDa protein appeared, which was recognized by polyclonal antibodies against both serpin-12 and HP14, suggesting that a covalent

Send correspondence to: Haobo Jiang, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, Telephone: (405)-744-9400, Fax: (405)-744-6039, haobo.jiang@okstate.edu.

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serpin-protease complex formed when serpin-12 inhibited HP14. Together, these data suggest that *M. sexta* serpin-12 inhibits HP14 to regulate melanization and antimicrobial peptide induction.

Graphical Abstract

Graph abstract:

I-TASSER model of *M. sexta* serpin-12 domain, along with the *N*-terminal extension rich in **Q/N**, **S/T**, and **P**. The P1 residue in the reactive center loop is in gray.

Keywords

insect immunity; hemolymph protein; serine protease cascade; melanization

1. Introduction

Melanization catalyzed by phenoloxidase (PO) is an immune response of insects, in which inactive prophenoloxidase (proPO) is converted to active PO by a proPO activating protease (PAP) in the presence of a cofactor of clip-domain serine protease homolog(s) (SPH) (Jiang et al., 2010; Park et al., 2010). PO hydroxylates monophenols to *o*-diphenols and oxidizes *o*-diphenols to *o*-quinones. Cytotoxic intermediates (*e.g.* 5,6-dihydroxyindole) and melanin sheaths sequester and kill the invading organisms at the site of infection (Kanost and Gorman, 2008; Zhao et al., 2011). ProPO activation is mediated by a branched serine protease cascade analogous to the blood coagulation system in mammals. When pathogens are encountered, members of the cascade pathway become active sequentially via specific proteolytic cleavages. Biochemical studies performed in *Manduca sexta* and *Tenebrio molitor* and genetic analysis in *Drosophila melanogaster* revealed striking similarities in

their proPO activation system constituents (Kanost and Jiang, 2015; Veillard et al., 2016). An initiating modular serine protease precursor becomes autoactivated upon interacting with pattern recognition proteins that bind to microbial surfaces. The modular serine protease then activates one or more downstream clip-domain serine proteases (CLIPs) (Kanost and Jiang, 2015; Jang et al. 2008; Buchon et al., 2009). In *M. sexta*, binding of β -1,3-glucan to β -1,3-glucan recognition proteins triggers the auto-proteolysis of hemolymph protease-14 (HP14) precursor at Leu³⁰⁴*Val³⁰⁵, HP14 cleaves CLIP proHP21 at Leu¹⁵²*Ile¹⁵³, HP21 activates CLIPs proPAP2 and proPAP3, and these PAPs generate active PO to catalyze melanization (Wang and Jiang, 2006 and 2007; Gorman et al., 2007; Jiang et al., 2003a and 2003b). Recognition of 2,6-diaminopimelic acid-peptidoglycan (PG) by the microbe-binding protein and peptidoglycan recognition protein-1 also leads to proHP14 auto-processing and system activation (Wang and Jiang, 2017). In *D. melanogaster* and *T. molitor*, Lys-type PG is recognized by peptidoglycan recognition protein-SA in the presence of Gram-negative bacteria-binding protein-1, which initiates the cascade by auto-activation of the modular serine protease precursors, which then activate CLIPs, resulting in the cleavage activation of pro-Spätzle and *T. molitor* proPO (Buchon et al., 2009; Park et al., 2007). The *M. sexta*, *D. melanogaster*, and *T. molitor* initiating modular proteases contain 4–5 low density lipoprotein-receptor class A repeats, two Sushi-like domains, and one serine protease (SP) catalytic domain (Wang and Jiang, 2010; Takahashi et al., 2015).

Protease inhibitors from the serpin superfamily contain a reactive center loop near the carboxyl-terminus, which acts as a bait for its cognate protease (Gettins, 2002). The P1 residue in the loop is the primary determinant of a serpin's inhibitory selectivity. When a target protease encounters the serpin, it cuts the peptide bond between the P1 and P1' residues, and this causes a major conformational change in the inhibitor, trapping the protease in an inactive acyl-enzyme covalent complex with the serpin. Serpins in the hemolymph of invertebrates regulate SPs activated during melanization, coagulation and other processes (Jiang et al., 2010; Kanost and Jiang, 2015; Meekins et al., 2017). In *M. sexta*, eight serpins inhibit CLIP proteases of the SP cascade leading to melanization and Toll pathway activation (Zhu et al., 2003; Tong et al., 2005; Zou and Jiang, 2005; Ragan et al., 2010; Suwanchaichinda et al., 2013; He et al., 2017). Here we report the cDNA cloning, expression profiling, and functional characterization of *M. sexta* serpin-12, with data indicating that serpin-12 regulates melanization by inhibiting the key initiating protease HP14 in hemolymph of *M. sexta* larvae.

2. Materials and methods

2.1. Insect rearing, immune challenge, and total RNA preparation

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Each 5th instar larva (day 2) was injected with a mixture of killed *Escherichia coli* XL1-Blue (2×10^7 cells) and *Micrococcus luteus* (20 μ g) (Sigma-Aldrich, M3770) suspended in 20 μ l of 0.85% NaCl. At 24 h post injection, induced hemolymph was collected from a cut proleg into a microfuge tube. Equal volumes of hemolymph from three larvae were pooled as one biological sample, and induced hemocytes (IH) and induced plasma (IP) were separated by centrifugation at $5,000 \times g$ for 15 min.

Plasma was stored in aliquots at -80°C for proPO activation assays. Control plasma (CP) and hemocytes (CH) were prepared in the same way from three day 2, 5th instar naïve larvae. Total RNA samples were extracted from hemocytes and from induced and naïve control fat body tissues (IF and CF) using Micro-to-Midi Total RNA Purification System (Invitrogen). To examine tissue distribution of the serpin-12 mRNA, integument, fat body, hemocytes, midgut, Malpighian tubules, muscle, nerve tissue, salivary gland, and trachea (each pooled from three insects) were dissected from day 3, 5th instar naïve larvae, rinsed in 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.4, prior to total RNA preparation. Similarly, fat body RNA samples (each pooled from three insects at the same stage) were isolated at various life stages ranging from day 0, 4th instar naïve larvae to day 3, adults. To test the protein level changes after immune challenge, *E. coli* cells (2×10^7 in 50 μl saline) were injected into day 2, 5th instar larvae. Hemolymph samples (each pooled from three insects at one time point) were collected at different times for separation by 10% SDS-PAGE, transfer to nitrocellulose membrane, and detection using 1:2000 diluted serpin-12 antiserum (Section 2.4), 1:3000 diluted alkaline phosphatase conjugated to goat-anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate, and nitro-blue tetrazolium (Bio-Rad). The incubation conditions were ambient temperature, 16 h (for primary antibody) and 4 h (for secondary antibody), and 1% dry milk in TBS (137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6).

2.2. cDNA cloning

Based on the sequences of EST contigs 3086, 3611, 5142 and 5627 (Zou et al., 2008) and their alignment with *Bombyx mori* serpin-12 cDNA, primers j117 (5'-CGAGCAATCTGGAATCTCGCT-3') and j119 (5'-TGGAAGCAATCCAGCTGTTGA-3') were synthesized to amplify a serpin-12 fragment from fat body cDNA pool in 25 cycles of 94°C , 30 s; 55°C , 30 s; 72°C , 30 s. The reaction mixture (1.0 μl) was used as template in a semi-nested PCR containing primers j118 (5'-TCTCGCTGCGTCGCCTTCTC-3') and j119 under the same PCR conditions. After agarose gel electrophoresis, the 329 bp product was recovered from the gel, confirmed by sequence analysis, and labeled with ^{32}P -dCTP (3000 Ci/mmol) using Multiprime DNA Labeling System (GE Healthcare Life Science). The cDNA probe was used to screen the induced *M. sexta* fat body cDNA library in λ ZAPII (Jiang et al., 2003a; Sambrook and Russell, 2001). Positive clones were purified to homogeneity and subcloned by *in vivo* excision of pBluescript phagemids. Plasmid DNA was isolated and completely sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems).

2.3. Multiple sequence alignment and phylogenetic analysis

A BLASTP search of GenBank (Database: NR, Organism: Insecta) was performed using *M. sexta* serpin-12 as a query. After a preliminary sequence alignment, representative full-length serpins from different orders of insects and *M. sexta* serpin-12 were selected for a more focused analysis. After phylogenetic analysis of all *D. melanogaster* serpins and ten *M. sexta* serpins (1I, 2-7, 9, 12, and 13) (Fig. S3a), we removed *D. melanogaster* serpins predicted to be non-inhibitory, not secreted, and those with a Lys or Arg at the putative P1 site (Reichhart, 2005) from further consideration as candidate serpins with likely

biochemical properties similar to *M. sexta* serpin-12. The remaining *D. melanogaster* serpins (28Da, 29F, 43Aa, 43Ac/Necrotic with a Leu, Met or Tyr at the P1 site) were included in the focused analysis. The retrieved sequences were aligned by MUSCLE using the following parameters: refining alignment, gap opening penalty = -2.9, gap extension penalty = 0, hydrophobicity multiplier = 1.2, maximum iterations = 100, clustering method (for iterations 1 and 2) = UPGMB, and maximum diagonal length = 24. The aligned sequences were converted to NEXUS format by MEGA (Kumar et al., 2016), and phylogenetic analyses were conducted using MrBayes v3.2.6 (Ronquist et al., 2012) under the default model with the setting “nchains=12”. MCMC (Markov chain Monte Carlo) analyses were terminated after the standard deviations of two independent analyses were <0.01. FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree>) was used to display the phylogenetic tree.

2.4. Production of *M. sexta* serpin-12 N and its polyclonal antibodies

To express an N-terminally truncated serpin-12 in insect cells, its coding region was amplified from the longest cDNA by PCR using primers j137 (5'-CCATGGGAATTCATTCGGGGCCTGCATC-3') and j143 (5'-TCAAGCATGCTCGAGTGTAGGATCCATTATTCTTGC-3') and ligated with pGEM-T DNA (Promega) before transformation. The underlined regions indicate the designed restriction sites for preparing expression constructs. After sequence validation, the *EcoRI*-*XhoI* fragment was subcloned into pMFH₆ (Lu and Jiang, 2008) and, in a separate experiment, the *NcoI*-*SphI* fragment was subcloned into H6pQE60 (Lee et al., 1994).

For baculovirus expression, the plasmid MsSpn12 N/pMFH6 was used to transform *E. coli* DH10Bac for *in vivo* transposition of the expression cassette. The resulting bacmid DNA was used to transfect Sf9 cells using Cellfectin (Invitrogen). Titer of the initial viral stock (V_0) was maximized through serial infections. Sf9 cells (2.0×10^6 /ml) in 500 ml Sf-900 II SFM medium were infected with the final stock at a multiplicity of infection of 10 and grown at 27°C for 96 h with gentle agitation. After centrifugation at $5,000 \times g$ for 15 min, the supernatant was collected, diluted with 500 ml distilled water containing 1 mM benzamidine, and incubated at 4 °C for 30 min. Following centrifugation at $12,000 \times g$ for 30 min, the cleared supernatant (1.0 L) was applied to a dextran sulfate-Sepharose column (40 ml) equilibrated in buffer A (10 mM potassium phosphate, 1 mM benzamidine, pH 6.4) and washed with 200 ml of the buffer. Bound proteins were eluted with a linear gradient of 0–1.0 M NaCl in buffer A (100 ml). The fractions (4 ml each) were collected and their aliquots (7 µl each) were subjected to 10% SDS-PAGE and immunoblotting with 1:2000 diluted anti-His5 antibody (Bio-Rad). Serpin fractions were pooled and applied to a column of 2 ml nickel-nitrilotriacetic acid agarose (Qiagen), equilibrated with buffer B (50 mM potassium phosphate, pH 8.0, 0.3 M NaCl, 10 mM imidazole, 0.005% Tween-20) and washed with 10 ml of buffer B. Bound proteins were eluted from the column with a linear gradient of 10–100 mM imidazole in buffer B (10 ml). Finally, tightly bound proteins were eluted with 5 ml of buffer B containing 250 mM imidazole. The fractions (1 ml each) were collected and analyzed as described above. The serpin-12 N fractions were pooled, concentrated, and buffer exchanged to 20 mM Tris-HCl, pH 7.5, 50 mM NaCl on Amicon-30 ultracentrifugal devices (Millipore). About 600 µg of the serpin-12 N was used to preparing a rabbit

polyclonal antiserum at Cocalico Biologicals Inc. and the remaining protein was stored at -80°C in aliquots.

For *E. coli* expression, JM109 cells harboring MsSpn12 N/H6pQE60 were grown at 37°C in 500 ml of 2×YT medium containing 100 µg/ml ampicillin. When A_{600} reached 0.7, isopropyl β -D-thiogalactoside was added to the culture at a final concentration of 1 mM to induce the serpin synthesis for 4–6 h at 37°C . The *E. coli* cells were pelleted by centrifugation at $5000\times g$ for 20 min at 4°C and resuspended in 20 ml of 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole, 0.005% Tween-20, 5% glycerol, and 1 mg/ml of lysozyme. After incubation at 4°C for 1 h with gentle shaking, the cells were sonicated on ice for 10 min, and soluble proteins were separated from cell debris by centrifugation at $65,000\times g$ for 1 h. The 6×His-tagged serpin-12 N in the supernatant was affinity purified on a column of 10 ml Ni^{2+} -nitrilotriacetic acid agarose beads according to the manufacturer's protocol for native proteins. The serpin fractions were pooled and dialyzed against pH 7.5, 20 mM Tris-HCl, 50 mM NaCl, concentrated on Centricon-30, and stored in aliquots at -80°C .

2.5. cDNA synthesis, quantitative RT-PCR, and immunoblot analysis

The total RNA samples (1.0 µg each) were incubated with 2.0 µl 5×iScript Reverse Transcription Supermix (Bio-Rad) in a 10 µl reaction at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. After 1:4 dilution, the cDNA samples (2.0 µl each) were incubated with 5 µl 2×iQ Taq Universal SYBR Green Supermix (Bio-Rad) and specific primers (5 pmol each) in 10 µl reactions on a CFX Connect Real-Time PCR Detection System (Bio-Rad). The thermal cycling conditions were: denaturation at 95°C for 30 s followed by 50 cycles of 95°C for 10 sec and 60°C for 30 sec. *M. sexta* ribosomal protein S3 (rpS3) cDNA (Jiang et al., 1996) was used as an internal control to normalize the samples using specific primers j055 (5'-CTCAGGCCGAGTCTTTGAGATACA) and j057 (5'-ACATAGTCATTGCATGGGTCACCG). Primers j146 (5'-GTGGGAGATTCCGTTTAGGGAACA-3') and j147 (5'-GCGGTAAGACGACGATAAGAGAGT-3') were used to specifically amplify the serpin-12 cDNA. To detect protein level changes, plasma samples were collected at 0, 1, 6, 12, 24, and 32 h after day 2, 5th instar larvae had been injected with 1×10^8 killed *E. coli* cells. Equal volume of the samples from three larvae at each time point were pooled and treated with SDS sample buffer. Following 10% SDS-PAGE, proteins were electrotransferred onto a nitrocellulose membrane for immunoblot analysis as described in *Section 2.1*.

2.6. Inhibitory activity assay and detection of serpin-protease complex

MsSpn12 N (200 ng/µl, 1.0 µl) from the insect cells and buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1.0 µl) control were separately incubated with freshly diluted proteases (100 ng/µl, 1.0 µl) and the buffer (8.0 µl) for 10 min at room temperature. Then, 150 µl of substrate solution was added to each reaction: 100 µM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide for assay of bovine α -chymotrypsin, human neutrophil cathepsin G (Athens Research & Technology), or proteinase K, 50 µM *N*-acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide for bovine trypsin, and 100 µM *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide for porcine pancreatic elastase (Athens Research & Technology). Unless specified, the substrates and

proteases in this section were all ordered from Sigma-Aldrich. Absorbance at 405 nm was monitored continuously for 10 min on a microplate reader (Molecular Devices), and one unit of activity is defined as $A_{405\text{nm}}/\text{min} = 0.001$. To detect a serpin-protease complex, the reaction mixture and controls were treated with 1×SDS sample buffer at 95 °C for 5 min. Protein samples were separated by 10% SDS-PAGE and subjected to immunoblot analysis using 1:2000 diluted serpin-12 antiserum as the primary antibody.

2.7. Determination of cleavage site in the serpin-12 N

Serpin-12 N (1.0 µg) was incubated with 0.5 µg cathepsin G for 10 min at room temperature in 20 µl of 20 mM Tris-HCl, pH 7.5, 5 mM CaCl₂, 0.001% Tween-20. The reaction mixture and negative controls of the serpin and protease alone were separately mixed with an equal volume of α-cyano-4-hydroxycinnamic acid matrix on a plate, dried, and subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry in the linear mode on a Voyager Elite mass spectrometer (PerkinElmer Life Sciences). After the spectra had been calibrated with bovine insulin as an external standard, the molecular mass of a peak in the serpin-protease mixture, which was absent in the control spectra was compared with calculated monoisotopic masses of the hypothetical carboxyl-terminal serpin-12 peptides to deduce the cleavage site in the serpin-12 N. Positive identification was called within the error range of 500 ppm.

2.8. Inhibition of proPO activation in plasma from naïve and induced *M. sexta* larvae

To test a possible effect of serpin-12 on proPO activation in hemolymph, control and induced plasma (CP and IP) were obtained from day 3, 5th instar larvae as described in *Section 2.1*. Half of the plasma samples were treated with equal volume of 100% saturated ammonium sulfate (pH 7.0) to remove most of the negative regulators (*e.g.* serpins, PO inhibitors) while keeping the proPO activation system intact (Jiang et al., 2003a; He et al., 2017). After centrifugation, the pellet was resuspended in the same volume of buffer (20 mM Tris-HCl, pH 7.5, 0.001% Tween-20), and stored as aliquots at –80 °C. These preparations of 0–50% saturated (NH₄)₂SO₄-fractionated plasma were named “0–50 CP” and “0–50 IP”. Aliquots (1.0 µl) of CP, IP, 0–50 CP, or 0–50 IP were incubated on ice for 5 min with 0–5.0 µg of the serpin-12 N from *E. coli* and the buffer to a final volume of 19 µl. One µl of the microbial elicitor (*M. luteus*, 1 µg) or buffer was added to the mixtures. After incubation for 60 min at room temperature (C) or 10 min (I), PO activities were measured as described previously (Jiang et al., 2003a). Similarly, effect of the serpin-12 N (2.5 or 5.0 µg) from *E. coli* on proPO activation was examined using IP treated with *S. aureus* (PG-*Sc*, 0.1 µg) or *E. coli* (PG-*Ec*, 1.0 µg) peptidoglycan, curdlan (10 µg), or zymosan (10 µg) for 10 min at 25 °C. The concentration-dependent inhibition of *M. sexta* proPO activation was also tested using the serpin-12 N from Sf9 cells. Aliquots of IP (1.0 µl) were incubated for 5 min at 25 °C with 0–0.6 µg of the serpin (1.0 µl) in 7.0 µl, 20 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, 0.001% Tween-20. Then, 1.0 µl of *M. luteus* (1.0 µg) was added and incubated at 25 °C for 10 min prior to assay of PO activity. IP samples with low basal PO activity and high proPO activation (Tong and Kanost, 2005) were selected for the assay.

2.9. Detection of changes in hemolymph protein size after *M. luteus* elicitation

To identify hemolymph proteins whose proteolytic processing is affected by the presence of serpin-12 N after stimulation of immune protease cascades, aliquots (1.0 µl) of CP, IP, 0–50 CP, and 0–50 IP were pre-incubated with the serpin (5.0 µg) or buffer (20 mM Tris-HCl, pH 7.5, 0.001% Tween-20) on ice for 5 min and then mixed with 1.0 µg of *M. luteus* or buffer at room temperature for 15 min (IP or 0–50 IP) or 60 min (CP or 0–50 CP). After treatment with 1×SDS sample buffer, the reaction mixtures were subjected to SDS-PAGE, electrotransfer, and immunoblot analyses using 1:2000 diluted rabbit polyclonal antisera against HP14, HP21, HP6, HP8, PAP1, PAP3, SPH1, SPH2, or proPO2 as the primary antibodies (Ji et al., 2004; Jiang et al., 1997, 2003b, 2005; Wang et al., 2001; Wang and Jiang, 2010; Yu et al., 2003).

2.10. Detection of serpin-12 protease complex in *M. sexta* plasma

15 µl of IP was mixed with 12.3 µl, 100% saturated (NH₄)₂SO₄ to achieve 45% saturation. After centrifugation, the supernatant was removed, and the pellet was dissolved in 30 µl, 20 mM Tris-HCl, pH 7.5. This preparation was named 0–45 IP. Buffer (1.5 µl) or insoluble PG from *S. aureus* (1.5 µg in 1.5 µl) were incubated with 0–45 IP for 30 min at room temperature. One third of the mixtures (10.5 µl) were subjected to 10% SDS-PAGE and immunoblot analysis using 1:2000 diluted rabbit antiserum against the serpin-12, HP14, or the HP14 protease domain (Wang and Jiang, 2010).

3. Results and discussion

3.1. cDNA cloning, structural features, and sequence comparisons of *M. sexta* serpin-12

We identified four *M. sexta* EST contigs in our pyrosequencing project (Zou et al., 2008), which encode overlapping protein sequences similar to *B. mori* serpin-12 (Zou et al., 2009). These contigs did not merge into a single contig due to limited read quality and low sequencing throughput. However, careful analysis of the contigs allowed us to design specific primers for amplification of a 344 bp cDNA fragment. Following sequence confirmation, the fragment was labeled for use as a probe to screen an induced larval fat body library. From 1.2×10⁵ plaques, we obtained four positives and subcloned the plasmids by *in vivo* excision. The longest cDNA of *M. sexta* serpin-12 contains a 121 bp 5′-noncoding region, a 1,458 bp open reading frame, and a 1,094 bp 3′-untranslated region including a 9 bp poly(A) tail (Fig. S1). The open reading frame encodes a 485-residue sequence with an 18-residue predicted secretion signal peptide. Calculated *M_r* and *pI* values of the mature serpin are 51,487 Da and 8.51, respectively.

A BLASTP search of GenBank using the serpin-12 sequence as query revealed over forty highest scoring sequences from various insects. Eleven of these sequences including *Drosophila* Necrotic, contain an 81–308-residue N-terminal extension followed by a serpin domain (Fig. S2). These atypical N-terminal extensions are rich in Gln, Asn, Pro and Ser/Thr, quite hydrophilic [GRAVY (grand average of hydropathy) score: −1.570 to −0.433], likely glycosylated, and so far only found in some lepidopteran and dipteran serpins. While sequence similarity is observed in this region among the homologs from closely related species, the extension does not seem to be conserved in terms of size and structure.

To investigate potential phylogenetic relationships among the insect serpins most similar to *M. sexta* serpin-12, we first aligned the sequences of all *D. melanogaster* serpins and the 10 *M. sexta* serpins with currently available functional data and constructed a neighbor-joining tree (Fig. S3a). Three likely orthologous groups were found (Ms3-Dm27A, Ms6-Dm88Ea/b-, Ms13-Dm28Dc-Dm42Dc), but no such relationship was apparent between *Manduca* serpin-12 and any *Drosophila* serpins. It appears that multiple groups of serpin paralogs have evolved in the lepidopteran and dipteran lineages. To try to identify *Drosophila* serpins that may be structurally and functionally similar to *Manduca* serpin-12, we selected the serpins predicted to be inhibitory, secreted, and having a hydrophobic amino acid residue at the putative P1 site in the reactive center loop. These candidate serpins (Dm28Da, Dm28F, Dm43Aa, and Dm43Ac/Necrotic) have a Leu, Tyr, Met, or Leu at the predicted P1 site, respectively. Dm28Da, Dm28F, and Dm43Aa all belong to a clade of 15 serpins from *Drosophila* but none from *Manduca* (Fig. S3a). We then aligned these four *Drosophila* serpins with representatives from five orders of insects, including Lepidoptera, Hymenoptera, Coleoptera, Phthiraptera, and Blattodea. The MrBayes tree derived from this alignment (Fig. S3b) revealed that the monophyletic group of lepidopteran serpins is closer to the louse and cockroach serpins than to the four *Drosophila* serpins. The hymenopteran and coleopteran serpins are closer to the dipteran serpins than to the lepidopteran ones. Due to low sequence conservation of insect serpins and incomplete genome information for many species, it is difficult to assign serpin orthologs from insect species that are not closely related. Thus, we cannot determine whether any of the four *D. melanogaster* serpins may have an orthologous relationship with *M. sexta* serpin-12.

The reactive center loop (RCL) of a serpin extends out from the protein surface and acts as a bait for its cognate protease. RCL sequences are hypervariable due to lack of structural constraints, but specific residues including the P1 residue preceding the scissile bond are selected to maintain the serpin's role as a specific regulator of its physiological target. Hence, we examined the aligned RCL sequences to find out whether the insect serpins with highest BLAST scores when searching with serpin-12 as query, and including the four *Drosophila* serpins selected as described above, share a conserved region. The predicted P2-P1*P1'-P2' residues, AL*SY (*, predicted scissile bond) is highly conserved in the lepidopteran serpins (Fig. 1). The conservation of L*S is also observed in 9 of the 13 non-lepidopteran serpins, including *D. melanogaster* Necrotic (Levashina et al., 1999) and Spn28Da (Scherfer et al., 2008) and *T. molitor* Spn40 (Jiang et al., 2009). Thus, 15 of these 19 insect serpins may inhibit hemolymph proteases with a chymotrypsin- or elastase-like specificity. *T. molitor* Spn93C has L*M determined as its P1 and P1' residues, allowing it to inhibit the modular serine protease from the beetle (Jiang et al., 2011). *Drosophila* Spn28Da has L*S as the P1 and P1' residues and resides in the same clade with Spn28F (Y*S) and Spn43Aa (M*S) (Fig. S3a). These three serpins all lack an N-terminal extension that is present in Necrotic and *Manduca* serpin-12. Together, these data suggest intriguing structural similarities of *M. sexta* serpin-12 and *Drosophila* Necrotic that may be relevant to their biological functions.

3.2. Immune inducibility, tissue specificity, and stage-dependence of serpin-12 gene expression

In our previous RNA-seq analysis of naïve and induced hemocytes and fat body mRNA (Gunaratna and Jiang, 2013), we found that *M. sexta* serpin-12 was highly expressed in fat body (CF: 204, IF: 960) and low in hemocytes (CH: 8, and IH: 51) and upregulated in both tissues after immune challenge. Our quantitative PCR analysis of the 5th instar larval hemocytes and fat body samples showed similar results, with strongly increased expression after immune induction, especially in fat body (Fig. 2a). Immunoblot analysis (Fig. 2d) showed that serpin-12 is present at a low level in the hemolymph of day 2, 5th instar larvae and its concentration greatly increased by 24 and 32 h after injection of bacteria, consistent with the major mRNA level increase (Fig. 2c). The induced expression in *M. sexta* serpin-12 is similar to the enhanced synthesis of *D. melanogaster* Necrotic and Spn28Da, *T. molitor* Spn40, and *Antheraea pernyi* serpin-12 after microbial exposure (Levashina et al., 1999; Scherfer et al., 2008; Jiang et al., 2009; Kausar et al., 2018). We further determined serpin-12 mRNA levels in several tissues and found that the serpin gene was highly expressed in fat body, moderately in nerve and trachea, and low in the other tissues (Fig. 2b). We examined serpin-12 mRNA levels in fat body tissues collected at various life stages (Fig. 2c). There is a small peak in the middle of 4th instar, another in the early 5th instar, a major increase before the onset of the wandering stage, then maintaining a relatively lower and constant level through the prepupal, pupal, and adult stages. Such infection-independent up-regulation of expression around the onset of wandering and pupation was also observed in other immunity-related genes, such as antimicrobial peptides and hemolin (He et al., 2015; Kanost et al., 2004).

3.3. Expression and purification of *M. sexta* serpin-12 N in insect and *E. coli* cells

We considered the low production of full-length Necrotic in *E. coli* (Robertson et al., 2003) and used the baculovirus-insect cell expression system to produce the serpin-12's core domain with truncation of the N-terminal 96 residues of the mature protein (GIH⁹⁷SGP...MDPT⁴⁶⁶LEHHHH HH, 41,895 Da, underlined region: cDNA encoded) (Fig. S1). After cell removal, the secreted serpin-12 N in the culture medium was first captured on a dextran sulfate-Sepharose column and eluted with a linear gradient of NaCl. The serpin fractions were detected by SDS-PAGE followed by immunoblot analysis using antibodies to the hexahistidine tag. The pooled fractions were further purified by nickel affinity chromatography. Approximately 1.5 mg of the serpin-12 N were isolated from 1.0 L culture, 0.6 mg of which was used as an antigen to generate polyclonal antibodies in a rabbit. The purified protein migrated as a 42 kDa band on 10% SDS-PAGE under reducing condition (Fig. 3a, *left* panel). There was a minor mobility increase (Fig. S4) following PNGase F treatment, indicating that Asn²⁹³, Asn⁴⁶⁹, or both are modified by N-glycans as predicted (Fig. S1). No mobility change was observed after O-glycosidase treatment (data not shown). Probably due to the N-terminal extension, serpin-12 in induced plasma migrated to the 52 Da position (Fig. 3a, *right* panel), close to the calculated mass of 51,487 Da. In other words, the natural protein was not likely O-glycosylated and the difference in migration from 42 to 52 Da was due to the extension. After storage at -80 °C for two months, half of the intact domain was cleaved at multiple positions after F⁴²⁴, A⁴²⁵, A⁴²⁶, V⁴²⁷, A⁴²⁸, L⁴²⁹, and S⁴³⁰ in the reactive center loop (Fig. S5). This result suggests minute

amounts of proteases from the insect cells slowly digested the exposed RCL of *M. sexta* serpin-12.

In order to solve the degradation problem, we also produced the serpin-12 N in *E. coli* as a soluble protein (MHHHHHHAMGIIH⁹⁷SGP...MDPT⁴⁶⁶LEHARAAEA, 42,935 Da) (Fig. S1) and purified about 7.0 mg of the serpin from 0.5 L culture. The purified protein remained mostly uncleaved and migrated as a single major band at a position expected for the theoretical mass of the serpin-12 N (Fig. 3b). The serpin preparation was stable after prolonged storage at -80 °C and, hence, used in the later functional tests.

3.4. Inhibitory activity and selectivity of *M. sexta* serpin-12 N

For testing possible inhibition, the serpin-12 N from the infected insect cells was incubated with five commercially available serine proteases with different specificity: bovine α -chymotrypsin, bovine pancreatic trypsin, proteinase K, human neutrophil cathepsin G, and porcine pancreatic elastase (Fig. 4). At molar ratios of about 1.19:1, 1.36:1 and 1.24:1, serpin-12 inhibited 70%, 85% and 94% of the chymotrypsin, elastase and cathepsin G activities, respectively. Consistent with the protease inhibition, 60–70 kDa covalent complexes of serpin-12 N and the proteases were detected by serpin-12 antibodies after these reactions (Fig. S6). In contrast, no inhibition or complex formation was observed in the reactions with trypsin or proteinase K (data not shown). Neither was any immunoreactivity detected in controls of the five proteases (data not shown). We examined the cleavage product of the serpin-12 N from *E. coli* generated by cathepsin G using MALDI-TOF mass spectrometry (Fig. 5). In the control spectra of the serpin-12 N or protease alone, there was no mass peak in the range of 5,000–5,500 Da. A new mass peak of 5,323.30 Da was detected in the mixture, matching the monoisotopic mass of peptide SYDEPSLYFRANKPFLAILWDNRSSI PLFMARIMDPTLEHARAAEA (5,323.68 Da). Thus, the proteolytic cleavage of serpin-12 by cathepsin G occurred between Leu⁴²⁹ and Ser⁴³⁰, as predicted on the basis of sequence alignment (Fig. 1). The side chain of Leu⁴²⁹ (*i.e.* P1 residue) fits well with the primary specificity pockets of chymotrypsin, cathepsin G and elastase, which favor cutting next to a hydrophobic residue.

3.5. Inhibition of *M. sexta* proPO activation by the serpin-12 N

To explore the physiological role of serpin-12, we examined its effect on proPO activation. The components of the proPO activation cascade are present in naïve larvae and also in insects that have previously been exposed to bacteria by injection. However, concentrations of some proteases, serpins, and recognition proteins in the cascade increase significantly as an innate immune response after exposure to bacteria (He et al., 2016), perhaps altering the system balance. Therefore we tested the effects of serpin-12 on proPO activation in different plasma preparations, including plasma from naïve control larvae (CP), “induced” plasma collect 24 h after injection of *M. luteus* (IP), and the 50% saturated ammonium sulfate precipitation fractions of these two types of plasma (0–50 CP and 0–50 IP), a treatment that removes most of the negative regulators (*e.g.* serpins) but keeps the proPO activation system intact (He et al., 2017). We mixed to induced plasma with different amounts of serpin-12 N and, after incubation, triggered activation of the proPO cascade with *M. luteus*. We observed a serpin-12 concentration-dependent decrease in proPO activation (Fig. 6d). A 50% decrease

in proPO activation was achieved with addition of 0.2 μ g serpin-12 N. We observed similar effects with the different types of plasma preparations (CP, 0–50 CP, IP, and 0–50 IP) (Fig. 6, a and b), with a consistent observation that increased serpin-12 concentration resulted in a lower level of proPO activation. We also examined the inhibitory effect on proPO activation after stimulation of plasma with microbial cell wall components, including peptidoglycan from *S. aureus* or *E. coli*, curdlan, or zymosan. Significant reductions in proPO activation in the presence of recombinant serpin-12 were observed in all these cases (Fig. 6c). These results suggest that serpin-12 functions to inhibit a key protease in the proPO activation cascade in *M. sexta*. The spontaneous melanization phenotype of the *Drosophila* Spn43Ac and Spn28Da mutants (Levashina et al., 1999; Scherfer et al., 2008) is consistent with their regulation of some steps in the proPO activation system as we observe here for serpin-12 in *M. sexta*. The serpin-12 ortholog in *A. pernyi* controls proPO activation and antimicrobial peptide production (Kausar et al., 2018).

3.6. Proteolytic processing of hemolymph proteins affected by the added serpin-12 N

To explore the mechanism by which serpin-12 suppresses proPO activation, we examined cell-free hemolymph samples, prepared in different ways to optimize detection of individual plasma proteins and their proteolytic cleavage products (CP, 0–50 CP, IP, and 0–50 IP), incubated first serpin-12 N or buffer as a control, and then activated the proPO cascade by addition of *M. luteus*. We hypothesized that addition of exogenous serpin-12 N would inhibit one or more proteases in the proPO activation cascade, leading to a decrease in processing of cascade members. SDS-PAGE and immunoblot analysis revealed the following changes (Fig. 7 for representative samples showing clearest results and Fig. S7 for the complete data set with all plasma preparations): 1) Levels of proHP6 (37 kDa) and proHP8 (38 kDa) considerably decreased after addition of bacteria, with appearance of a band consistent with the size of their activated catalytic domain, but the zymogen bands remained at similar levels in the presence of serpin-12 N (Fig. 7 b and c). The cleavage of proHP6 (by proHP1* or an unknown HP) (He et al., 2017) and proHP8 (by HP6) (An et al., 2009) are likely indirectly affected by serpin-12 N, because the proteolytic activation sites of these enzymes are after basic residues, not a hydrophobic residue as occurs in the serpin-12 P1 position. 2) Similar results were observed for proPAP1 and proPAP3, with less proteolytic processing of the zymogen in the presence of serpin-12 N (Fig. 7 d and e). This observation is consistent with the observed lack of HP6 activation, since HP6 is an activator of proPAP1 (An et al., 2009). Note that the cleavage of proPAP3 may occur at a position upstream of the known proteolytic activation sites, resulting a heavy chain larger than the catalytic domain (37 kDa) and a light chain smaller than the dual clip domains (21 kDa) (Jiang et al., 2003b). 3) The processing of proSPH1 and proSPH2 was enhanced upon exposure to *M. luteus* and suppressed by addition of serpin-12 N (Fig. 7 f and g). As their cleavage sites are next to Arg residues (Yu et al., 2003), this is likely an indirect effect of serpin-12 N, which is not expected to inhibit proteases with trypsin-like specificity; 4) Serpin-12 N decreased the activation cleavage of proPO2 (Fig. 7h), consistent with the decrease in PO activity in the presence of this serpin (Fig. 6). 5) In plasma, proHP14 was detected as bands at 75 kDa, and a 67 kDa N-terminally truncated form previously observed (Wang and Jiang, 2006). After treatment of 0–50 IP with *M. luteus*, a faint band at about 48 kDa was detected by HP14 antibody (Fig. 7a), larger than the 45 kDa regulatory chain of

proHP14 (Wang and Jiang, 2010). The levels of proHP14 (75 and 67 kDa) did not change much. One possible explanation is that the catalytic chain of HP14 formed a covalent complex with a serpin and migrated to a position close to the 75 kDa proHP14 on the gel.

3.7. Evidence for the formation of a serpin-12-HP14 complex

To test a hypothesis that serpin-12 regulates the proPO cascade by inhibiting the initiating protease HP14, we carried out an experiment to detect the presence of a complex between serpin-12 and HP14 in a plasma preparation that contains the proPO cascade members. When we treated the 0–45% $(\text{NH}_4)_2\text{SO}_4$ fraction of IP with peptidoglycan from *S. aureus* (PG-*Sa*) and probed immunoblots with serpin-12 antibody, we observed the 52 kDa serpin-12 band and the appearance of a 71 kDa band (Fig. 8a, *left* panel), whose intensity increased after the microbial stimulation. This band, not detected in the PG-*Sa* only (data not shown), disappeared and likely shifted to a position of about 100 kDa under non-reducing condition (*right* panel). Similar band intensity changes at around 70 and 100 kDa were observed using antibodies specific for the HP14 catalytic domain (Wang and Jiang, 2010) (Fig. 8b). Antibodies against the entire HP14 recognized the 45 kDa regulatory and 30 kDa catalytic chains (Fig. 8c, *left* panel), which migrated to approximately 100 kDa location under non-reducing condition (*right* panel). While these results together supported the detected formation of an HP14-serpin-12 complex, three problems affected the unequivocal identification of these bands: 1) The 75 and 67 kDa isoforms of HP14 and proHP14 (Wang and Jiang, 2006), and various posttranslational modifications of serpin-12 influenced gel mobility (Fig. 8a); 2) Even after $(\text{NH}_4)_2\text{SO}_4$ fractionation, abundant hemolymph proteins (e.g. apolipoprotein-II and storage proteins) at 70–80 kDa caused band compression, making it difficult to determine protein sizes; 3) The large size and inefficient transfer of serpin-HP complexes, limited resolving power of the gel in the high M_r region, and incomplete linearization of polypeptides in the absence of dithiothreitol further compromised the interpretation. Note that *M. sexta* serpin-12 (52 kDa) is converted to 46, 44 and 40 kDa forms, indicating its proteolysis at multiple sites by other HPs (Fig. 8a). Under non-reducing condition, the complex of serpin-12 main portion (46 kDa) and HP14 catalytic domain (30 kDa) is predicted to be associated with the HP14 regulatory chain (45 kDa) through a disulfide bond and likely corresponded to the band at around 100 kDa.

Our results support a conclusion that serpin-12 regulates the proPO activation system in *M. sexta* by inhibiting the initiating serine protease in the pathway, HP14. The autoactivation site of the HP14 precursor is GTEL*VLGG (Wang and Jiang, 2006), and HP14 can cleave proHP21 at ADDL*IIGG (Wang and Jiang, 2007), both activities with cleavage after a P1 Leu residue. Serpin-12 has a Leu residue at the P1 position in its RCL. Other known proteases in the PO activation cascade, HP21, HP6, HP8 and PAP1–3, are trypsin-like enzymes that cut their substrates after an Arg or Lys residue (Kanost and Jiang, 2015). HP14 is the only chymotrypsin-like enzyme known so far to exist in the proPO activation system. Inhibition by recombinant serpin-12 blocks proteolytic processing of proteins downstream from HP14 in the PO cascade, consistent with its effect on HP14 as the first protease in the pathway. Hemolymph contains a protein of size and immune-reactivity consistent with a covalent complex of HP14 and serpin-12. Therefore, on the basis of this evidence, we suggest that HP14 is a physiological target of *M. sexta* serpin-12. Besides melanization,

proHP14 activation induces antimicrobial peptide synthesis and proHP6 activation (Wang and Jiang, 2017). Since HP6 activates proHP8, and HP8 activates proSpätzle-1 to induce antimicrobial peptide production (An et al., 2009 and 2010), we further suggest that the serpin-12 regulates a Toll pathway in *M. sexta*. *M. sexta* serpin-12 and *D. melanogaster* Necrotic have in common two unusual structural similarities, an amino-terminal extension and a Leu residue at P1 of the RCL, which are lacking in most insect serpins. Both of these serpins regulate proPO activation. We speculate that secreted insect serpins with these characteristic features of serpin-12 are candidates for inhibitors of the initiating modular proteases such as HP14, at the top of immune protease cascades in hemolymph.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PO and proPO	phenoloxidase and its precursor
PAP	proPO-activating protease
PG	peptidoglycan
HP	hemolymph protease
CLIP	clip-domain serine protease (SP) or serine protease homolog (SPH)
CF	CH, CP, IF, IH, and IP, fat body (F), hemocytes (H), and plasma (P) from day 3, 5 th instar control (C) naïve larvae or induced (I) larvae challenged with a mixture of <i>E. coli</i> and <i>M. luteus</i>
0–50 CP/IP	0–50% saturated (NH ₄) ₂ SO ₄ -fractionated CP/IP
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
RCL	reactive center loop
rpS3	ribosomal protein S3
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Highlights

- cDNA cloning, structure features, and expression profiles of *M. sexta* serpin-12
- Closer evolutionary relationship to Necrotic than many other *Drosophila* serpins
- Inhibition of chymoelastase-like proteases by the serpin (P1: Leu⁴²⁹)
- Broad impacts on members of the proPO and Toll activation system
- Probable regulation of *M. sexta* hemolymph protease-14 by the serpin-12

	14	1	
Ms12	IDER GG SAAAATSFAAVAL LSY	DEPSLYFRANKPFLAIL	
Ha	IDEK GG SAAAATAFAAVAL LSY	DEPSIRFRVNRPFIAVL	
Bm12	IDEK GG SAAAATSFGVVAL LSF	DDPAVVFKANRPFLAVL	
Cs2	IDEQ GG VAAAATSFGVVAL LSY	DDPSVIFRANRPFLAVL	
Dp	VDEQGLSATAATSFAAIAL LSY	EDPPALFQANRPFLAVL	
Px	IDEQ GG TAAAATGFAAVAL LSG	DDDTIYFKADRPFLAIL	
Zn	VNEK GG TASAATAILVATL SLVV	NPDELQFNANHPFLAII	
Pc	IDEE GS VASAATGIFFEVTSFI	PTDNLDFIADHPFLFFI	
Cc	VNEEG SE AAAATGLMMRPLSLNWSPPKPLEFRVDRPFPLATI		
Ar	VNEEG SE AAAVTVVQIVALSAISFDQPPKTIKVNRPYLYMI		
Oa	VNEEG SE AAAATGIVIEVLSI	SIPETFHVDRPFHYEI	
Ap	VNEEG SE AAAATGVYAVTL SLII	DPNKLEFVADHPFIALL	
At	VNEEG SE AAAATAIMVDSL SFV	EPETESFKADHPFLLYL	
Dm28Da	IDEE GG SAGSASASPIRG LSDY	ATSVVTFTVNSPFVFMI	
Dm28F	VNEEGAEAAAATALLFVRY SMPM	PSSQMFVNADHPFAYVI	
Tm40	VAEQGVEAAAADYYLIALP LS	GPPKSIKMFKANHPFIFYI	
Tm93C	VNEYG TR AAAVNTAVVI PLM	GSSAQRIADQPFIFFI	
DmNec	VGEAG TE ASAASYAKFV PLSL	PPKPTEFVANRPFVFAV	
Dm43Aa	VNEIGCEAAGASYAAGVP MSL	PLDPKTFVADHPFAFII	
	: * * *	: :	: : : * * : :

Fig. 1. Aligned RCL sequences of *M. sexta* serpin-12 and its close relatives from other insects

As described in *Section 2.3*, preliminary studies yielded representative serpins from different orders of insects for the focused analysis. These include *Aethina tumida* XP_019867147.1, *Agrilus planipennis* XP_018329668.1, *Athalia rosae* XP_012262999.1, *Bombyx mori* serpin-12, *Cephus cinctus* XP_015609143.1, *Chilo suppressalis* serpin-2, *Danaus plexippus* OWR48572.1, *D. melanogaster* serpin-28Da, 28F, 43Ac, 43Ac/Necrotic, *Helicoverpa armigera* XP_021198929.1, *M. sexta* serpins-12, *Orussus abietinus* XP_012271558.1, *Pediculus corporis* XP_002424080.1, *Plutella xylostella* XP_011551761.1, *Tenebrio molitor* serpin-40, 93C, *Zootermopsis nevadensis* XP_021938028.1. The first letters of their genus and species names are used to distinguish the proteins. The predicted P19–P19' region of *M. sexta* serpin-12 and the corresponding parts in the other serpins are taken from the alignment of their complete sequences. The putative P14 (Gly/Ser/Thr) and P1 (Leu) residues are in bold. The experimentally determined P1 residue is in red font. Positions with strictly (*) and highly (:) conserved residues are indicated below.

Fig. 2. Immune inducibility, tissue specificity, and developmental profile of *M. sexta* serpin-12 expression

(a) As described in *Section 2.5*, total RNA samples from the control (C) and induced (I) hemocytes (H) and fat body (F) were analyzed by qRT-PCR using serpin-12 specific primers, along with an internal standard of rpS3. CH and CF: hemocytes and fat body from naïve larvae; IF and IH: hemocytes and fat body from the induced larvae at 24 h after bacterial injection. **(b)** Total RNA samples of integument (I), fat body (F), hemocytes (H), midgut (G), Malpighian tubules (Mt), muscle (M), nerve tissue (N), salivary gland (Sg), and trachea (T) from day 3, 5th instar naïve larvae were analyzed. **(c)** Fat body total RNA samples from day 0–5, 4th instar naïve larvae (4th), days 0–5, 5th instar naïve larvae (5th), days 1–7, wandering larvae (W), days 1, 15, 24 pupae (P), and day 3, adult (A) were examined. After normalization with rpS3, the relative levels of serpin-12 mRNA are adjusted based on the lowest (1.0) in each group (**a**, **b**, or **c**) and shown in the bar graph (mean $\pm$ SD, $n = 3$). **(d)** Cell-free hemolymph samples (2 μ l each), collected at indicated times after day 2, 5th instar larvae had been injected with 1×10^8 killed *E. coli*, were subjected to 10% SDS-PAGE and immunoblot analysis using 1:2000 diluted *M. sexta* serpin-12 antiserum as the primary antibody.

Fig. 3. Electrophoretic analysis of the affinity purified *M. sexta* serpin-12 N expressed in baculovirus-infected insect cells (a) and *E. coli* (b)
(a) The purified protein (0.5 μ g) from Sf9 cells was separated by 10% SDS-PAGE followed by Coomassie brilliant blue staining (*left* panel). For comparison, full-length serpin-12 in 1.0 μ l induced plasma was resolved by 7.5% SDS-PAGE and detected by immunoblotting (*right* panel). **(b)** The purified protein (1.5 μ g) from *E. coli* cells. Positions and sizes (in kDa) of the protein M_r markers are indicated. *, intact serpin; **, cleaved serpin.

Fig. 4. Inhibition of enzymatic activities of various serine proteases by *M. sexta* serpin-12 N
 As described in *Section 2.6*, aliquots the serpin-12 N from the insect cells or buffer were separately incubated with α -chymotrypsin (Chy), trypsin (Tryp), proteinase K (ProK), cathepsin G (CathG), or porcine pancreatic elastase (PPE). The amidase activities were measured using specific substrates. Residual activities (mean $\pm$ SD, $n = 3$) relative to the controls of enzyme only (100%, gray bar) were plotted as open bars to show inhibition in the serpin reactions.

Fig. 5. MALDI-TOF mass spectrometric analysis of the peptide generated in the reactions of *M. sexta* serpin-12 N and cathepsin G

A representative single accumulation spectrum is presented for each of the three samples [brown, Spn12 N from *E. coli*; blue, cathepsin G (CathG) only; green, Spn12 N and CathG]. The mass spectrometer was calibrated with bovine insulin and its performance was monitored using the external standard. Mass value of the new major peak in the reaction mixture is labeled on top of the peak.

Fig. 6. Inhibition of proPO activation in hemolymph samples by *M. sexta* serpin-12 N from *E. coli* (a–c) and Sf9 (d) cells

(a, b) As described in Section 2.8, aliquots of the control and induced plasma (CP and IP) and their $(\text{NH}_4)_2\text{SO}_4$ fractions (0–50 CP and 0–50 IP) were pre-incubated with 0–5.0 μg of the serpin-12 N from *E. coli* and stimulated with 1.0 μg of *M. luteus* or buffer (negative control) for 1 h (CH) or 10 min (IH) at room temperature prior to PO activity measurement. (c) Aliquots of IP were pre-incubated with buffer or the serpin-12 N (left, 5 μg; right, 2.5 μg) from *E. coli* and the mixtures were individually treated with buffer (control), *S. aureus* PG (left panel), *E. coli* PG, curdlan, or zymosan (right panel) for 10 min at 25 °C before the activity assay. (d) The concentration-dependent inhibition of proPO activation was also tested using the serpin-12 N from the virus-infected insect cells. Aliquots of IP was pre-incubated for 5 min at 25 °C with buffer or 0–0.6 μg of the serpin. Then 1.0 μl of *M. luteus* (1.0 μg) was added to the mixtures and incubated for 10 min at room temperature prior to the PO activity assay. All the activities (mean ± SEM) are plotted in the bar graphs.

Fig. 7. Changes of the hemolymph proteases (HPs), serine protease homologs (SPHs), and proPOs in plasma samples from *M. sexta* larvae

As described in *Section 2.9*, aliquots of CP, IP, 0–50 CP and 0–50 IP were incubated with buffer (lane 1), *M. luteus* (lane 2), or first with the serpin-12 N from *E. coli* and then with *M. luteus* (lane 3). Following 7.5%, 10% or 12% SDS-PAGE and electrotransfer, the blots were separately incubated with 1:2000 diluted polyclonal antisera against the HPs, PAPs, SPHs, and proPO2 as indicated in the bottom of each panel. The representative results for HP6 (panel **b**) and PAP1 (panel **d**) are from CP, for SPH1 (panel **f**) from IP, for HP8 (panel **c**) from 0–50 CP, and for HP14 (panel **a**), PAP3 (panel **e**), SPH2 (panel **g**) and proPO2 (panel **h**) from 0–50 IP. Positions and sizes of the M_r markers are shown on the *left*. The precursor proteins (*), putative serpin-protease complexes (◆), cleavage products (arrowhead) for heavy (*h*) and light (*l*) chains or catalytic (*c*) and regulatory (*r*) domains, and the nonspecific band of the serpin-12 N (○) are indicated.

Fig. 8. Detection of an SDS-stable complex containing *M. sexta* serpin-12

As described in *Section 2.10*, 0–45 IP was separately incubated with buffer (1.5 μ l, lane 1) and insoluble PG from *S. aureus* (lane 2), resolved by 10% SDS-PAGE, and analyzed by immunoblotting using 1:2000 diluted antiserum against the serpin-12 (**a**), HP14 protease domain (**b**), or full-length HP14 (**c**) as the primary antibody. The precursor proteins (“*”), cleavage products (arrowhead) for heavy regulatory (*r*) and light chains or catalytic (*c*) domains, and possible serpin-protease complex (●) are indicated.