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Serpin-9 and -13 regulate hemolymph proteases during immune responses of *Manduca sexta*

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Abstract

Serpins are a superfamily of proteins, most of which inhibit cognate serine proteases by forming inactive acyl-enzyme complexes. In the tobacco hornworm *Manduca sexta*, serpin-1, -3 through -7 negatively regulate a hemolymph serine protease system that activates precursors of the serine protease homologs (SPHs), phenoloxidases (POs), Spätzles, and other cytokines. Here we report the cloning and characterization of *M. sexta* serpin-9 and -13. Serpin-9, a 402-residue protein most similar to *Drosophila* Spn77Ba, has R³⁶⁶ at the P1 position right before the cleavage site; Serpin-13, a 444-residue ortholog of *Drosophila* Spn28Dc, is longer than the other seven serpins and has R⁴¹⁰ as the P1 residue. Both serpins are mainly produced in fat body and secreted into plasma to function. While their mRNA and protein levels were not up-regulated upon immune challenge, they blocked protease activities and affected proPO activation in hemolymph. Serpin-9 inhibited human neutrophil elastase, cathepsin G, trypsin, and chymotrypsin to different extents; serpin-13 reduced trypsin activity to approximately 10% at a molar ratio of 4:1 (serpin: enzyme). Serpin-9 was cleaved at Arg³⁶⁶ by the enzymes with different specificity, but serpin-13 had four P1 sites (Arg⁴¹⁰ for trypsin-like proteases, Gly⁴⁰⁶ and Ala⁴⁰⁹ for the elastase and Thr⁴⁰⁴ for cathepsin G). Supplementation of induced cell-free hemolymph (IP, P for plasma) with recombinant serpin-9 did not noticeably affect proPO activation, but slightly reduced the PO activity increase after 0–50% ammonium sulfate fraction of the IP had been elicited by bacteria. In comparison, addition of recombinant serpin-13 significantly inhibited proPO activation in IP and the suppression was stronger in the fraction of IP. Serpin-9- and -13-containing protein complexes were isolated from IP using their antibodies. Hemolymph protease-1 precursor (proHP1), HP6 and HP8 were found to be associated with serpin-9, whereas proHP1, HP2 and HP6 were pulled down with serpin-13. These results indicate that both serpins regulate immune proteases in hemolymph of *M. sexta* larvae.

Graphical Abstract

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Keywords

protease inhibitor; insect immunity; phenoloxidase; serine protease cascade; hemolymph proteins; reactive center loop

1. Introduction

Some insect defense mechanisms are mediated by serine proteases and regulated by serpins (Kanost and Jiang, 2015; Meekins et al., 2017; Veillard et al., 2016). Upon infection, the immune system can recognize surface features of the invading microbes through pattern recognition receptors, transmit the signal of danger via extracellular serine protease (SP) pathways, and cleave precursors of serine protease homologs (SPHs), phenoloxidases (POs), and cytokines (*e.g.* spätzles, stress responsive peptides) (Jiang et al., 2010; Kurata, 2010; Park et al., 2010). The proteolytically activated proteins then trigger melanization, Toll pathway, antimicrobial peptide synthesis, cellular encapsulation, and other responses to eliminate the pathogens. During melanogenesis, POs produce reactive intermediates that kill a broad spectrum of microorganisms as well as host cells (Zhao et al., 2007 and 2011). ProPO activation and other serine protease-mediated immune responses are regulated to enhance pathogen destruction and reduce cytotoxicity to the host.

A framework of immune serine protease system has been elucidated in a biochemical model insect *Manduca sexta*, which includes 4 pattern recognition receptors, 8 hemolymph (serine) proteases (HPs), 2 SPHs, and 6 serpins (Kanost and Jiang, 2015; Suwanchaichinda et al., 2013; Wang and Jiang, 2017; Yang et al., 2016). Specific binding of peptidoglycans or β -1,3-glucans by their receptors induces autoactivation of proHP14, HP14 activates proHP21, HP21 generates proPO activating proteases (PAP2 and PAP3), PAP2/3 activates proPOs in the presence of a high M_r complex of SPH1 and SPH2. In another branch, proHP1 in an active conformation (*) (He et al., 2017) may cleave proHP6 to form HP6; HP6 converts proHP8 to HP8 that activates pro-Spätzle; HP6 also cleaves proPAP1 to form PAP1 that activates proPOs in the presence of the SPH complex. Some of the HPs are inhibited by serpin-1, -3 through -7. For example, proHP1* forms 90 kDa complexes with serpin-1, -4, -9, -13 and small amounts of serpin-3, -5, -6 (He et al., 2017).

Serpins are a superfamily of 45–50 kDa proteins, most of which form covalent complexes with cognate SPs to regulate, for instance, human blood coagulation (Gettins, 2002) and insect innate immunity (Meekins et al., 2017). In the *M. sexta* genome, there are 32 serpin genes, some encoding multiple variants to perform different functions (Kanost et al., 2016). Serpin-1J formed a complex with PAP3 *in vitro* (Jiang et al., 2003); Serpin-1A, -1E and -1J inhibit HP8 (An et al., 2009); Serpin-1K forms a complex with a midgut chymotrypsin (Ragan et al., 2010). Serpin-3 efficiently blocks proPO activation in the hemolymph and forms complex with PAP1 and PAP3 *in vitro* (Zhu et al., 2003). It also affects the Toll pathway activation via HP8 inhibition (Christen et al., 2012). Serpin-4 and -5 suppress components of the proPO activation pathway, including proHP1*, HP6, HP21 and others (An and Kanost, 2010; Tong et al., 2005; Tong and Kanost, 2005). Serpin-6 specifically inhibits PAP3 to control proPO activation *in vitro*. PAP3, HP8, proPO, and a few other proteins associated with serpin-6 were pulled down by affinity beads conjugated with the serpin antibodies and identified by mass spectrometry (Wang and Jiang, 2004; Zou and Jiang, 2005). Like serpin-1J, -3 and -6, serpin-7 forms an SDS-stable complex with PAP3 and regulate melanization (Suwanchaichinda et al., 2013). Evidence further indicates that other inhibitors may also modulate HPs. Serpin-X-HP14 and serpin-Y-HP8 complexes were detected in the larval hemolymph (Tong et al., 2005). Fifteen cDNA contigs discovered in a pyrosequencing project encode polypeptides similar to regions of insect serpins (Zou et al., 2008). In this paper, we report the cDNA cloning of serpin-9 and -13 using information from four of the contigs, expressed the two recombinant serpins, and characterized their roles in the larval hemolymph.

2. Materials and Methods

2.1. Insect rearing, immunization, plasma collection, RNA preparation, and cDNA synthesis

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Each of day 2, 5th instar larvae was injected with a mixture of *Escherichia coli* (1.3×10^7 cells), *Micrococcus luteus* (13 µg), and curdlan (13 µg) in 20 µl H₂O. Induced hemolymph was collected from cut prolegs of the larvae 24 h later and centrifuged at $5,000 \times g$ for 5 min to remove hemocytes and obtain induced plasma (IP). The plasma samples were aliquoted and stored at –80°C for proPO activation, serpin-protease complex isolation, and other experiments. Similarly, control plasma was prepared from day 3, 5th instar naïve larvae. To increase sensitivity of the proPO activation assay, a known volume of the induced hemolymph was treated with equal volume of 100% saturated ammonium sulfate to remove most of the negative regulators (*e.g.* serpins, PO inhibitors) while keeping the proPO activation system intact in the (NH₄)₂SO₄ fraction. After centrifugation, the pellet was resuspended in the same volume of 20 mM NaCl, 20 mM Tris-HCl, pH 7.5, aliquoted, and stored at –80°C as “0–50% (NH₄)₂SO₄ fractionated IP” or “0–50 IP”.

Total RNA samples were isolated from hemocytes (H) and fat body (F) of the induced (I) and naïve or control (N or C) larvae using Trizol Reagent (Life Technologies). Integument, Malpighian tubule, midgut, muscle, nerve tissue, salivary gland, and trachea were dissected from 5–10 of day 3, 5th naïve larvae for RNA extraction. In the developmental series, fat

body and cell-free hemolymph were collected from naïve larvae and pupae at various life stages (3–6 insects per stage) for RNA isolation and immunoblot analysis, respectively. For cDNA synthesis, the total RNA samples (1 µg each) were incubated with 1× iScript Reverse Transcription Supermix (Bio-Rad) in a 10 µl reaction at 25°C for 5 min, 42°C for 60 min, and heated at 95°C for 5 min to inactivate the enzyme.

2.2. cDNA cloning of *M. sexta* serpin-9 and -13

BLASTX (<http://www.ncbi.nlm.nih.gov/>) was employed to search the non-redundant protein sequence database with the four putative serpin contigs (Zou et al., 2008), confirm their identity, and find conserved regions to design primers: for contig 1483, j120 (5'-TACTTCAGAGGAGCG TGGA) and j122 (5'-ACATGGAGTATCTTCCATCGT); for contig 3437, j123 (5'-ATCCAGGC GTCTATGTTGATTC) and j125 (5'-TGAACATGTAGAATGGGGAGTC); for contig 4324, j126 (5'-CGTCGGCGAGGCTCTCAGTG) and j128 (5'-ATGGCATCTTCATAGTGTAGTC); for contig 4857, j129 (5'-ACACGGTACGTTTCGCGCAATGC) and j131 (5'-TGGAGTCTCAGGAC TCGCTGGA). After a PCR test using CH, CF, IH and IF cDNA as templates, the induced fat body (IF) sample was selected to amplify the cDNA fragments. The reactions (25 µl) contained 100 ng of cDNA, 10 pmoles of each primer, 2.5 U Taq polymerase, and 1×buffer with 4 dNTPs. The thermal cycling conditions were 35 cycles of 94°C, 30 s; 50°C, 30 s; 72°C, 50 s. Products at the expected sizes (196, 144, 211, and 216 bp) were recovered and labeled with [α -³²P]-dCTP (3000 Ci/mmol) using Multiprime DNA Labeling System (GE Healthcare Life Science). The four probes were used individually to screen *M. sexta* induced fat body cDNA library according to (Sambrook and Russell, 2001). Positive plaques were purified to homogeneity and subcloned by *in vivo* excision of the 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

Amino acid sequences of the serpins of *M. sexta*, *Bombyx mori*, *Anopheles gambiae*, and *Drosophila melanogaster* were retrieved from NCBI and aligned using 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 trees.

2.4. Recombinant expression and polyclonal antibody generation

The serpin-9 cDNA was used as a template for PCR amplification using specific primers: J139 (5'-CCATGGGAATTCCGGCTCGGGGCTTCAT) and J140 (5'-TAAGCTTACTCGAGGCTTGCTTAGAGTAC). Primers J141 (5'-CCCATGGGAATTCAAGATGAACAATAC) and J142 (5'-

AAGCTTACTCGAGTCCTTTTGTGGGATCGAA) were used to amplify the serpin-13 cDNA by PCR. The underlined regions in these primers are incorporated restriction sites for preparing expression constructs. Following T/A cloning and sequence validation, the inserts were retrieved by *Nco*I-*Hind*III double digestion and subcloned into the same sites in H6pQE60 expression vector (Lee et al., 1994). The recombinant plasmids encode an amino-terminal hexahistidine tag fused with a serpin domain. *E. coli* JM109 cells harboring the plasmid were grown at 37°C in 800 ml of LB medium containing 100 µg/ml ampicillin. When A₆₀₀ reached 0.8–1.0, isopropyl β-D-thiogalactoside was added to the culture at a final concentration of 0.75 mM to induce serpin synthesis for 4 h at 37°C. The *E. coli* cells were spun down at 5000×*g* for 20 min at 4°C and resuspended in 40 ml of pH 7.5, 50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, 0.005% Tween-20, 5% glycerol, and 1 mg/ml of lysozyme. Following incubation at 4°C for 1 h with gentle shaking, the cells were sonicated on ice for 8 min and soluble proteins were separated from cell debris by centrifugation at 15,000×*g* for 30 min. The native serpins were purified by affinity chromatography using Ni²⁺-nitrilotriacetic acid (NTA) agarose beads (Qiagen) according to the manufacturer's protocol. Serpin-9 or -13 in the elution fractions was pooled and dialyzed against pH 7.5, 20 mM Tris-HCl, 50 mM NaCl overnight at 4°C, concentrated on Centricon-30, aliquoted, and stored at –80°C for inhibition tests. To generate serpin-9 and -13 polyclonal antibodies, insoluble proteins in the cell debris were dissolved in 6 M urea and the recombinant serpins in the solution were then isolated by Ni-NTA chromatography under denaturing condition. The purified proteins were dialyzed against pH 7.5, 20 mM Tris-HCl, 50 mM NaCl and concentrated to about 1 mg/ml. Around 0.6 mg of each serpin was separated by 10% preparative SDS-PAGE, briefly stained with Coomassie blue, and destained with ddH₂O. The serpin-9 and -13 bands were cut, smashed, and emulsified with adjuvants for preparation of rabbit polyclonal antiserum at Cocalico Biologicals.

2.5. Analysis of serpin-9 and -13 mRNA and protein levels

To profile serpin-9 and -13 expression, cDNA samples (each equivalent to starting with 50 ng total RNA) were incubated with 1× iTag Universal SYBR Green Supermix (Bio-Rad) and specific primers (0.5 µM each) in triplicate in each reaction (10 µl). The primers were: j037 (5'-CATGATC CACTCCGGTGACC) and j038 (5'-CGGGAGCATGATTTTGACCTTAA) for rPS3, j150 (5'-TAGT GTCGAGGGAGCTGGTTTATG) and j151 (5'-AAGTACAGAGCGTCGATCATCACC) for serpin-9, and j154 (5'-GTCCGAAACACGTACCAGGA ACTA) and j155 (5'-TCCAAGTAATCACGGAA CACGTCTG) for serpin-13. The thermal cycling conditions were: denaturation at 95°C for 2 min, followed by 40 cycles of 95°C 10 s and 60°C 30 s. After the PCR was complete on a CFX Connect Real-Time PCR Detection System (Bio-Rad), melting curves of the products in all reactions were examined to ensure proper shape and T_m values. The mRNA levels were normalized against the internal control of ribosomal protein S3 (set at 1.00) using corresponding Ct values for the same cDNA samples and the relative mRNA levels were calculated as 2^{–Ct}, where Ct = Ct_{serpin} – Ct_{rpS3}.

To detect possible changes in serpin-9 and -13 protein levels at different stages, hemolymph samples of at least two insects at the same life stage were pooled, diluted, and treated with the SDS sample buffer. The protein samples (each equivalent to 1.0 µl plasma) were resolved

by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. After blocking with 3% dry milk in Tris-buffered saline (TBS) for 1 h, the membranes were incubated with 1:1000 diluted serpin-9 or -13 polyclonal antiserum overnight. After TBS washing, 1:1000 goat-anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) in TBS was incubated with the membranes for 3 h at room temperature prior to TBS washing and development using AP Conjugate Substrate Kit (Bio-Rad).

2.6. Inhibition of protease activity by serpin-9 and -13

Several commercially available serine proteases were tested for possible inhibition by serpin-9 and -13, which included trypsin, chymotrypsin, cathepsin G, proteinase K, human neutrophil elastase (HNE), and porcine pancreatic elastase. Recombinant serpin-9 and -13 at different concentrations were separately mixed with these enzymes in 20 mM Tris-HCl, pH 8.0 for 10 min at room temperature. Same volume of the buffer was used as a control to replace serpin. Residual amidase activities were measured at 405 nm on a VersaMax microplate reader (Molecular Devices) using 100 μ M of different substrates in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, *N*-acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR*p*NA) for trypsin; *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF*p*NA) for chymotrypsin, proteinase K and cathepsin G; *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide (AAPL*p*Na) for the elastases. One unit activity is defined as $A_{405}/\text{min} = 0.001$. To compare the extents of inhibition of different proteases by serpins at specific molar ratios, activities of the control groups were adjusted to 100 and percentages of the residual activities in the test groups were calculated and plotted.

2.7. Determination of the cleavage sites in serpin-9 and -13

To determine the position of scissile bond in serpin-9, 4.0 μ g of the purified inhibitor was incubated with 1.0 μ g of cathepsin G, thrombin, HNE, or factor Xa in 20 μ l of 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 5 mM CaCl₂, 0.001% Tween 20 at room temperature for 10 min. The reaction mixtures and negative controls (serpin-9 and individual proteases) were subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry in the linear mode (Jiang et al., 2003). The cleavage site in serpin-13 was determined by the same method using trypsin and the four proteases above. Serpin-13 and trypsin were used as negative controls. Molecular mass of a peak in a serpin-protease mixture, which was absent in the control spectra of serpin and protease only, was compared with calculated monoisotopic masses of the carboxyl-terminal peptides to deduce the cleavage site in that serpin. Positive identifications were called within the error range of 500 ppm.

2.8. Inhibition of proPO activation in induced plasma by *M. sexta* serpin-9 and -13

Aliquots of induced plasma (IP, 1 μ l) or its ammonium sulfate fraction (0–50 IP, 1 μ l, *Section 2.1*) were incubated with buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, positive controls), serpin-9 or serpin-13 at different concentrations on ice for 10 min and then with *M. luteus* (5 μ g) at room temperature for 10 min. Induced plasma and its fraction in the absence of *M. luteus* were used as negative controls. PO activities in the test and control groups were measured using 150 μ l of 2.0 mM dopamine as described previously (Jiang et al., 2003).

2.9. Isolation and characterization of serpin-9, -13, and their complexes with HPs in larval plasma

To isolate the serpins and their acyl-complexes with certain HPs, 10 ml of cell-free plasma were collected from naïve 5th instar larvae. *M. luteus* (1 µg/µl) and 1-phenyl-2-thiourea (about 1 mM) were added to the control plasma (CP for serpin-9) or induced plasma (IP for serpin-13) to stimulate the serine protease system and, meanwhile, inhibit PO that generates reactive compounds for protein crosslinking. After 30 min incubation at room temperature, a cocktail of protease inhibitors (cOmplete ULTRA, Roche Diagnostics, 1.0 ml) was added to block the protease activities. The mixtures were centrifuged at 5000×*g* for 10 min at 4°C to remove debris. The supernatants were loaded onto a column containing 1.2 ml Protein A-Sepharose coupled with serpin-9 or -13, as described before (Zou and Jiang, 2005). After washing with 1 M NaCl in TBS to remove unrelated proteins, bound proteins were eluted with 0.5 ml aliquots of 50 mM glycine-HCl (pH 2.5) into tubes containing 50 µl of the neutralizing buffer (1.0 M Tris-HCl, pH 8.0). The elution fractions were separated by SDS-PAGE for Coomassie blue staining and immunoblot analysis using the respective serpin antibodies. After fractions containing the most serpin-protease complexes were located, aliquots of the combined fractions were subjected to 10% SDS-PAGE and immunoblot analysis. Polyclonal antisera (1:1000 diluted) against *M. sexta* serpin-9, -13, HP1–3, 5–9, 12, 13, 15, 17, 18, 21, 22, 27, 28, SP112, and PAP1–3 were used as the primary antibodies to separately detect possible presence of the antigens in the eluted proteins.

3. Results

3.1. cDNA cloning, structural features, and orthologs of *M. sexta* serpin-9 and serpin-13

Our analysis of immunity-related expressed sequence tags (Zou et al., 2008) revealed 15 *M. sexta* cDNA contigs encoding polypeptide sequences similar to regions of insect serpins. Based on the genome sequence (Kanost et al., 2016), we now know that contigs 1483 and 4857 encode two different parts of the serpin-9 whereas contigs 3437 and 4324 encode two regions of the serpin-13. Since such relationships were unknown when the contigs became available, we designed a pair of primers for each of the four and amplified the cDNA fragments for use as probes to screen 1.2×10⁵ plaques of an induced fat body cDNA library, hoping to obtain four different serpin cDNAs. Instead, we obtained seven positive clones (B1, B2, B6 and B8 using probe 1483; E1, E2 and E5 using probe 4857) for the serpin-9 and seven (C5, C8, C10 and C11 using probe 3437; D1, D2 and D6 using probe 4324) for the serpin-13. As shown in Fig. S1, the open reading frames in the longest cDNA clones of serpin-9 and -13 encode polypeptides of 402 and 448 residues, with calculated masses of 45,558 and 51,154 Da and isoelectric points of 5.00 and 5.81, respectively. After removal of the 20- and 21-residue signal peptide, the mature proteins are predicted to be glycosylated at N¹⁰⁶, S¹¹⁵ (in serpin-9), S³³, S³⁵, N²⁶⁴, N³⁵², S³⁶⁰, S³⁶⁴ (in serpin-13) and secreted into extracellular space. Detection of the two inhibitors in plasma (see below) is consistent with the premises. Multiple sequence alignment of the published *M. sexta* serpins (Fig. S2) indicates that residues 347–378 of serpin-13 (NSNTIN³⁵²KTEDELIS³⁶⁰KKNS³⁶⁴DQINETGFVEMLNN) may form an extended region rich in hydrophilic residues (72%) and modified by *N*- and *O*-linked glycosylation between helix I and beta strand 5A. In contrast, the rest of the serpin domain contains 48%

hydrophilic residues and is, therefore, much higher in hydrophobicity than the extended portion: -0.258 versus -1.113.

The reactive center loops of serpins vary dramatically in length and sequence. As shown in Fig. 1A, there are 30, 33, 30, 25, 25, 28, 25, 25 and 23 residues between the absolutely conserved Gly (P15) and Phe residues in serpin-1J, -2 through -7, -9 and -13, respectively. The residues near the cleavage site differ greatly, except for the P1 residue. *M. sexta* serpin-1J, -3 through -7, -9 and -13 all have a positively charged P1 residue (Arg or Lys).

The phylogenetic tree (Fig. 1B) shows that serpin-9 is the ortholog of *B. mori* serpin-7. Both proteins are members of a serpin clade that also includes *D. melanogaster* Spn77Ba and *A. gambiae* serpin-8. *M. sexta* serpin-4, -5, -7, -9 and *B. mori* serpin-4, -5, -7, -8, -14, -31 and -32 are located in the same clade, indicative of sister relationships in each moth – a sister group denotes the closest relatives of an operational taxonomic unit in a phylogenetic tree. This is consistent with the proposed evolutionary history of the seven silkworm serpin genes (Zou et al., 2009). On the other hand, *M. sexta* serpin-13, *B. mori* serpin-13 and *D. melanogaster* Spn28Dc are 1:1:1:3 orthologous to *A. gambiae* SRPN4, 5 and 6. This agrees with the previous finding that *Drosophila* Spn28Dc and *Anopheles* SRPN4–6 belong to a single phylogenetic clade (Abraham et al., 2005).

3.2. Changes in mRNA and protein levels of serpin-9 and -13

In the RNA-seq analysis of naïve and induced hemocytes and fat body mRNA (Zhang et al., 2011), we found that serpin-9 and -13 were highly expressed in fat body and low in hemocytes (reads numbers for serpin-9 in CF: 59, IF: 149, CH: 5, and IH: 4; for serpin-13 in CF: 53, IF: 111, CH: 2, IH: 1). After normalization, the I/C ratios of mRNA in fat body and hemocytes for serpin-9 were 1.3 and 1.0 after immune challenge while those for serpin-13 were only 1.1 and 0.6, respectively. Our qPCR analyses of the 5th instar larval hemocytes and fat body samples showed the similar trend (Fig. 2A): serpin-9 mRNA levels remained unchanged in fat body and decreased in hemocytes after the immune challenge whereas serpin-13 transcripts decreased considerably in fat body and more so in hemocytes. Relatively low read numbers of the RNA-seq analysis and batch differences between the qPCR and RNA-seq samples may be responsible for the value differences. We also determined mRNA levels of the two serpins in several tissues (Fig. 2B) and found their tissue distributions were similar: serpin-9 and -13 genes were highly expressed in fat body (2.3-fold higher than rpS3), moderately in nerve (both serpins), trachea (serpin-13) and hemocytes (serpin-13), and low in other tissues. This resemblance in expression profiles was also observed in the 52 RNA-seq datasets, with serpin-9 and -13 located next to each other in the same cluster (data not shown). Since fat body is a major site for the serpin synthesis, we then examined the serpin-9 and -13 mRNA levels in fat body tissues collected in various life stages (Fig. 2C). Their expression profiles generally agreed with each other, which had a peak in the early 5th instar larvae, another before the onset of wandering stage, and the third around pupation. This kind of infection-independent up-regulation of expression was also observed in other immunity-related genes (e.g. antimicrobial peptides) (He et al., 2015).

Since fat body mRNA levels in general do not correlate well with their protein abundances in *M. sexta* plasma (He et al., 2016), we examined levels of the two serpins in hemolymph

samples, each pooled from 2 or 3 insects. Serpin-9 level did not increase at 24 h after the immune challenge and decreased after *M. luteus* stimulation (Fig. 2D, *lower* panel). In comparison, serpin-13 level decreased at 24 h after the challenge but did not change much following *M. luteus* treatment. Serpin-9 was most abundant in 4th instar larvae, became lower in the 5th instar feeding larvae, and disappeared in the early-to-middle wandering stage (Fig. 2D, *upper* panel). Following a reappearance in the late wandering stage, serpin-9 level peaked in the bar stage, decreased after pupation, and reemerged in pupae on day 9. The serpin-13 level changes were not as dramatic. It was most abundant in feeding larvae, decreased to lower levels during wandering stage, and disappeared in the pupal stages tested. Because proteins are functional molecules, physiological roles of the serpin-9 and -13 should be interpreted after dynamics of the mRNA and protein abundance changes at various developmental stages are taken into consideration.

3.3. Expression of *M. sexta* serpin-9 and -13 and characterization of their inhibitory activities

In order to understand biochemical functions of the serpin-9 and -13, we produced them as soluble proteins in *E. coli*. Since the plasmid constructs encoded a hexahistidine tag at the amino-terminus, we purified the recombinant serpins by Ni²⁺ affinity chromatography and resolved the samples by 10% SDS-PAGE under reducing conditions (Fig. 3). The proteins migrated as major bands to positions close to the theoretical masses of serpin-9 (44,035 Da) and -13 (48,455 kDa). The protein bands were recognized by their specific polyclonal antibodies.

To study their inhibitory selectivity, we measured the residual activities of several commercially available SPs after they had been separately incubated with buffer, serpin-9 or serpin-13 (Fig. 4). At molar ratios of 1.34:1 and 1.06:1, serpin-9 inhibited 44.6% HNE and 56.2% cathepsin G ($p < 0.05$). The HNE inhibition by serpin-9 was more complete (90%) at a ratio of 2.68:1. On the other hand, the purified serpin-13 inhibited 42.6% trypsin, 37.7% HNE, and 48.6% cathepsin G ($p < 0.05$) at molar ratios of 1.58, 1.00 and 1.19 to 1, respectively. The concentration-dependent inhibition of trypsin by serpin-13 (80% at 3.95:1) was more explicit than by serpin-9 (10% at 2.00:1). Neither serpins inhibited proteinase K, chymotrypsin or porcine pancreatic elastase, not even at higher molar ratios of serpin to serine protease (data not shown).

3.4. Determination of the cleavage sites in serpin-9 and -13

To further characterize the inhibition reactions, we examined the cleavage products of serpin-9 or -13 generated by cathepsin G, thrombin, HNE, factor Xa or trypsin using MALDI-TOF mass spectrometry (Fig. 5). In the control spectra of serpin-9, -13 or the proteases alone, there was no mass peak in the range of 4,000–5,000 Da, except for trypsin that autolyzes. A new mass peak of 4,163–4,164 Da was detected in the mixture of serpin-9 and cathepsin G/thrombin/HNE/factor Xa, which is identical to the theoretical mass (4164.08 Da) of MMPEQFVANKPFLFMIVDKQIDVM LFTGAYSKPSLE, the carboxyl-terminal peptide released from proteolytic cleavage of serpin-9 at Arg³⁶⁶ by the enzymes. In the reaction mixture of serpin-13 and cathepsin G/thrombin/factor Xa/trypsin, we detected another new mass peak at 4,260–4,261 Da. The experimental values are nearly identical to

the calculated mass (4261.11 Da) of the carboxyl-terminal peptide released from serpin-13 after cleavage between Arg⁴¹⁰ and Ser⁴¹¹ by these enzymes. The 4,260 Da peak detected in the serpin-13-cathepsin G reaction was much smaller than the 4,856 Da peak, which may represent the 4858.47 Da fragment generated by cutting between Thr⁴⁰⁴ and Ser⁴⁰⁵. In the serpin-13-HNE reaction, two new peaks were detected at 4,415 and 4,599 Da. They match the theoretical masses (4417.21 and 4601.33 Da) of the carboxyl-terminal peptides generated by HNE cleavage next to Ala⁴⁰⁹ and Gly⁴⁰⁶, consistent with the elastase's substrate specificity. The differences between these observed and calculated masses are all within the error range of the instrument (500 ppm, or ± 2.0 Da for a 4,000 Da peptide).

3.5. Inhibition of the proPO activation system by serpin-9 and -13

To test whether or not serpin-9 and -13 regulate the extracellular serine protease system that mediates immune responses in *M. sexta*, we added various amounts of serpin-9 or -13 to the induced plasma and, after incubation for 10 min on ice, triggered proPO activation with *M. luteus* for 10 min at room temperature. The activation was reduced by serpin-13 from 27.4 U of PO activity to about 14.9 U ($p < 0.01$) but not by serpin-9 (Fig. 6A). To increase sensitivity of the assay, we performed 0–50 (NH₄)₂SO₄ fractionation to preferentially reduce endogenous negative regulators while keeping the activation system largely intact. After the treatment, PO activity levels in the negative controls (plasma only) became higher while those in the positive controls (plasma with *M. luteus* only) reduced considerably (Fig. 6B). In the presence of serpin-13 (1.0 and 2.0 μ g), half and two-third of the proPO activation were blocked. Small but statistically significant reductions of proPO activation were observed after *M. luteus* elicitation in the presence of exogenous serpin-9 (2.4 and 3.2 μ g). Taken together, these results indicated that serpin-9 and -13 do regulate melanization. The effect of serpin-9 appears to be smaller than serpin-13 and other serpins in whole plasma from the immune challenged larvae.

3.6. Isolation and characterization of the serpin-HP complexes formed in larval plasma

To better understand their regulatory functions, we tested whether or not serpin-9 and -13 form covalent complexes with HPs in hemolymph by immunoaffinity chromatography and immunoblot analysis. We first collected larval plasma, activated it with *M. luteus*, and used the serpin antibodies to capture the inhibitors and their acyl-complexes with SPs. As shown in Fig. 7A, proteins eluted from the serpin-9 antibody column contained a 90 kDa serpin-9-proHP1* complex (He et al., 2017), 70–75 kDa serpin-9-protease complexes, 44 kDa serpin-9, and a large amount of 35 kDa immunlectins that bind Sepharose beads (Christen et al., 2012). While the 90 kDa complex was characterized recently, the 70–75 kDa bands were also recognized by HP1, HP6 and HP8 antibodies but not by HP2, 3, 5, 7, 9, 12, 13, 15, 17, 18, 21, 22, 27, 28, SP112, and PAP1–3 antibodies (data not shown). ProHP1* and HP6 are involved in proPO activation while HP8 is responsible for the proteolytic activation of *M. sexta* proSpätzle-1 (An et al., 2009; An and Kanost, 2010; Yang et al., 2016).

Similarly, serpin-13 antibodies isolated two proteins migrating as broad bands around 55 and 50 kDa, which may represent the intact and cleaved serpin, respectively. The difference between the apparent molecular mass of 55 kDa and the calculated mass of 48,274 Da is likely due to glycosylation of the natural serpin (Fig. 7B), as predicted in Fig. S1. Proteins

eluted from the serpin-13 antibody column also included 35 kDa immulectins, 70–75 kDa serpin-13-protease complexes, and 90 kDa serpin-13-proHP1* complexes. The 90 kDa complex confirmed the result of a similar pulldown experiment using the HP1 polyclonal antibodies (He et al., 2017). The 75–70 kDa faint bands were weakly recognized by HP2 and HP6 antibodies, respectively. ProHP1* and HP6 are members of the proPO activation system and HP2 is implicated in the same process (He and Jiang, unpublished results). The antibodies against HP3, 5, 7–9, 12, 13, 15, 17, 18, 21, 22, 27, 28, SP112, and PAP1–3 did not detect any clear band in the range of 70 and 75 kDa in the eluted protein samples (data not shown).

4. Discussion

Pyrosequencing of transcriptomes can provide useful leads to discover process-related genes in organisms lacking known genome (Zhang et al., 2011; Zou et al., 2008). By taking advantage of the sequence information, we isolated cDNA clones coding for two novel serpins in *M. sexta*, a well-known model species for insect immunobiochemical investigations (Jiang et al., 2010; Kanost and Jiang, 2015). We then examined the expression of serpin-9 and -13 at the mRNA and protein levels. Using soluble proteins produced in *E. coli*, we studied inhibitory activity and selectivity of the serpins using a panel of commercially available SPs and MALDI-TOF mass spectrometry. In the concentration-dependent tests using induced plasma and its fraction (0–50 IP), we established the regulatory roles of serpin-9 and -13 in proPO activation. The functional data were further supported by the identification of serpin complexes containing proHP1*, HP6 and other HPs involved in the immune responses.

4.1. Structural, functional, and evolutionary relationships of the serpins

Phylogenetic analysis showed that *M. sexta* serpin-9 is orthologous to *B. mori* serpin-7, *D. melanogaster* Spn77Ba, and *A. gambiae* SPRN8 (Fig. 1B). Based on the current study, we suggest that the uncharacterized serpins also regulate immune responses in other insects including the silkworm, fly, and mosquito. *M. sexta* serpin-4, -5, -7 and -9 and their 1:1 orthologs *B. mori* serpin-4, -5, -7 and -32 are located in the same clade of the phylogenetic tree and the *M. sexta* serpins inhibit proHP1*, HP6, HP21 and PAP3 (He et al., 2017; Suwanchaichinda et al., 2013; Tong et al., 2005). This clade also include *B. mori* serpin-8, -14 and -31, whose 1:1 orthologs are found in the *M. sexta* genome (data not shown). It appears that, before divergence of the last common ancestor of *M. sexta* and *B. mori*, seven serpin genes had already arisen from a single intron-less gene (Zou et al., 2009). According to the observed functions of *M. sexta* serpin-4, -5, -7 and -9, we suggest that the product of their ancestral gene had immuno-regulatory roles and it would be interesting to find out whether or not the other sister genes also encode serpins that regulate the *M. sexta* immune system.

M. sexta serpin-13 is orthologous to *B. mori* serpin-13, *D. melanogaster* Spn28Dc, and *A. gambiae* SRPN4–6. Spn28Dc expression is highly induced after injury and it regulates melanization in hemolymph and tracheal compartments of the larvae and adults (Scherfer et al., 2008). Spn28Dc may control the initial release of PO but it is unclear how a serine

protease inhibitor may affect the process. *A. gambiae* SRPN6 participates in the clearance of malaria parasites by inhibiting melanization and promoting parasite lysis (Abraham et al., 2005). Functions of these orthologs are consistent with the immuno-regulatory roles of *M. sexta* serpin-13. On the other hand, there is a notable difference between the lepidopteran serpin-13s and their dipteran orthologs, the 31/32-residue region rich in hydrophilic residues. It is unclear whether or not this insert has a functional significance at present, which seems to adopt a disordered structure between helix I and β -strand 5A.

4.2. Inhibition of serine proteases with different specificity by serpin-9 and -13

The reactive center loop of a serpin determines its inhibitory selectivity (Gettins, 2002). Since the residue before the cleavage site directly interacts with the primary specificity pocket of a target protease, property of the P1 residue largely governs the rate and stoichiometry of inhibition. In some cases, one serpin (*e.g.*, α 1-protease inhibitor, α 2-antiplasmin, miropin) inhibits several proteases with different specificity (Enghild et al., 1993; Ksiazek et al., 2015). These enzymes can cleave the inhibitor at a single or multiple peptide bonds in the loop, suggestive of an induced fit between the serpin and proteases. How fast the cleaved loop inserts into β -sheet A of the inhibitor affects the partitioning between stable inhibition and substrate cleavage (Lawrence et al., 2000) and, thus, the extent of inhibition at a certain ratio of serpin to protease. We believe such scenarios should be common due to the hypervariability of reactive center loops in serpins, and this study provides two examples. In the first example, different P1 residues in the reactive site loop seem to be responsible for the broad selectivity of serpin-13. *M. sexta* serpin-13 inhibited thrombin, factor Xa, trypsin, proHP1*, HP2, and HP6 (Figs. 4, 5, and 7), which is consistent with Arg⁴¹⁰ located at the confirmed P1 site and trypsin-like specificity of these proteases. The same serpin also inhibited HNE, which prefers small, hydrophobic residues and cleavage of the reactive site loop indeed occurred next to Ala⁴⁰⁹ and Gly⁴⁰⁶. In contrast, cathepsin G cut between Thr⁴⁰⁴ and Ser⁴⁰⁵ and, to a lesser extent, between Arg⁴¹⁰ and Ser⁴¹¹, which do not seem to agree with the protease specificity and observed inhibition (Fig. 4A).

In comparison, thrombin, factor Xa, proHP1*, HP6 and HP8 all have trypsin-like specificity that fit well with Arg³⁶⁶ at P1 site of serpin-9 (Fig. 5). Perhaps, the rate of trypsin cleavage at Arg³⁶⁶ is much faster than the loop insertion, serpin-9 acts mainly as a substrate (Fig. 4A). On the other hand, a marginal fit of Arg³⁶⁶ with the specificity pockets of cathepsin G (chymotrypsin-like, cutting next to Phe and Tyr) and HNE (preferring Ala, Gly, and others) may be enhanced by Pro³⁶⁵, favoring the formation of stable serpin-9-protease complexes. Pro³⁵⁷ at P2 site of human α 1-protease inhibitor makes a contribution to its inhibition of elastase, trypsin, and chymotrypsin.

4.3. Exploration of *M. sexta* serpin-9 and -13's immune-regulatory roles

While inhibitory activities of the serpins are worth exploring using commercially available proteases, it is more pertinent to reveal their physiological roles by identifying their endogenous targets. To this end, the data presented herein suggest that serpin-13 is a stronger inhibitor of proPO activation than serpin-9 (Fig. 6). The two serpins are relatively specific: among the twenty HPs predicted as trypsin-like, only three were detected in the

complexes with each inhibitor using the twenty-one HP antibodies (Fig. 7). In light of our findings on their broad inhibitory selectivity of serpin-13, we speculate that it may inhibit chymotrysin- or elastase-like HPs (*e.g.* HP14). HP14 acts as an initiation enzyme of the proPO activation system, which receives the signal of fungal and bacterial infection through β -1,3-glucan recognition protein-1/2, microbe-binding protein, and peptidoglycan recognition protein-1 (Kanost and Jiang, 2015; Wang and Jiang, 2017). If serpin-13-HP14 does exist, the additional control of HP14 and HP2 (Fig. 7B) by serpin-13 may help explain why more serpin-9 complexes with proHP1* and HP6 (Fig. 7A) did not result in better inhibition of proPO activation (Fig. 6). We would like to identify additional target proteases using antibodies against chymotrysin- or elastase-like HPs (Cao et al., 2015) or by mass spectrometric analysis of the 70–75 kDa regions. It would also be interesting to test whether or not addition of serpin-9 or -13 may affect the proteolytic activation of precursors of cytokines (*e.g.* Spätzles, stress responsive peptides), as a step toward understanding the generation, function, and regulation of immune signaling molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AS	ammonium sulfate
CF, IF, CH, and IH	fat body (F) and hemocytes (H) from control (C, <i>i.e.</i> naïve) and immune challenged (I) larvae
HP	hemolymph (serine) protease
IP	cell-free hemolymph (<i>i.e.</i> plasma) from induced larvae
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
NTA	nitrilotriacetic acid
PO and proPO	phenoloxidase and its precursor
PAP	proPO activating protease
SP	serine protease
SPH	non-catalytic serine protease homolog
TBS	Tris-buffered saline

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Highlights

- cDNA cloning, structure features, and expression patterns of *M. sexta* serpin-9 and -13
- Serpin-9 (P1: Arg³⁶⁶) inhibits serine proteases with different specificities
- Serpin-13 uses Arg⁴¹⁰, Ala⁴⁰⁹, Gly⁴⁰⁶, Thr⁴⁰⁴ as P1 residues for different proteases
- Serpin-9 forms small amounts of covalent complexes with proHP1*, HP2, and HP6
- Serpin-13 inhibits proHP1*, HP6 and HP8 to suppress the proPO activation system

Fig. 1. Multiple sequence alignment of reactive center loop regions in the known *M. sexta* serpins (A) and phylogenetic relationships of the entire serpins from the four insects (B)

The complete amino acid sequences of *M. sexta* serpin-1 through -7, -9 and -13 were first aligned (Fig. S2) as described in *Section 2.3*. Their reactive center loop regions are extracted from the alignment and shown in panel A, with the P14 and P1 residues marked by vertical bars. The P1 residues are in **bold red** font. Positions with strictly, highly, moderately conserved residues and gaps are labeled “*”, “:”, “.”, and “-”, respectively. In panel B, sequences of the serpin proteins in the mosquito *A. gambiae* (Ag), fly *D. melanogaster* (Dm), silkworm *B. mori* (Bm), and *M. sexta* (Ms) were aligned (data not shown) for constructing the phylogenetic tree. Probability for branches are indicated near the branching points, with “*” representing 100. *M. sexta* serpin-9 and -13 are indicated with *closed red* circles, and the other seven with *open red* circles. The branches containing *M. sexta* serpin-9 and -13 are in *blue* and *red*, respectively.

Fig. 2. *M. sexta* serpin-9 and -13 mRNA and protein levels in samples from different life stages (A) Immune inducibility. Serpin-9 and -13 transcript levels in the control (C) and induced (I) hemocyte (H) and fat body (F) RNA samples were measured by quantitative (q) PCR, calculated based on Ct values of the test (serpin) and control (rpS3) genes in the three biological replicates (3 larvae per sample) and plotted in the bar graphs (mean $\pm$ SD, $n = 3$). Values of the means are indicated on top of the error bar. For A–C, serpin-9, gray bar; serpin-13, maroon bar. (B) Tissue specificity. Serpin-9 and -13 transcript levels in the RNA samples of hemocytes (H), fat body (F), integument (I), midgut (Mg), Malpighian tubule (Mt), muscle (Mu), nerve tissue (N), salivary gland (S), and trachea (T) from day 3, fifth instar naïve larvae were determined by qPCR under the same conditions. (C) Developmental profiles. The serpin-9 and -13 mRNA levels in fat body from naïve larvae and pupae at different life stages were determined by qPCR. “4”, 4th instar, day 1; “5-e/m/l”, 5th instar, day 1/2/3; “W-e/m/l”, wandering, day 1/2/5; “Bar”, brown-bar stage before pupation; “P-1/4/7/9”, pupa, day 1, 4, 7 or 9. *Note:* While the relative serpin-9 and -13 mRNA levels are juxtaposed in panels A–C, they cannot be directly compared based on strict terms. (D) Protein abundances. Serpin-9 and -13 levels in cell-free hemolymph (1.0 μ l/lane) from the naïve larvae and pupae (see panel C) were tested by immunoblotting using diluted antiserum against serpin-9 (gray arrowhead) or -13 (maroon arrowhead) as the first antibody. Similarly, gel mobility of the serpins in the control and induced larval plasma (CP and IP) before and after *M. luteus* elicitation (MI+) was also examined.

Fig. 3. SDS-PAGE and immunoblot analyses of the recombinant serpin-9 and -13

The two affinity purified proteins (1.0 μ g for the stained gel and 50 ng for the blot) were separated by 10% SDS-PAGE and visualized by Coomassie staining (panel **A**) or immunoblotting (panel **B**) using diluted antiserum against the serpin-9 or -13 as the primary antibody. Positions and sizes of the M_r markers are shown on the *left*. The intact serpin-9 and -13 bands are labelled with *gray* and *maroon* arrowheads, respectively.

Fig. 4. Inhibitory activities of *M. sexta* serpin-9 and -13

(A) Inhibition of six commercially available proteases by *M. sexta* serpin-9 (upper, gray) or -13 (lower, maroon). Aliquots of the buffer or purified recombinant serpins (1.0 μ g) were separately incubated with the proteases (1 μ g) in pH 8.0, 20 mM Tris-HCl for 10 min at room temperature. The amidase activities (mean $\pm$ S.D., $n = 3$) were measured using 150 μ l of the peptide substrates, as described in Section 2.5. Enzyme activities in the control (Ctrl) reactions of protease alone were adjusted to 100% for calculating residual activities (%) in the corresponding reactions containing serpin-9 or -13 also. “*” and “**” indicates significant ($p < 0.05$ and < 0.001) decrease relative to the controls, respectively. Serpin-protease molar ratios are listed below trypsin (Tryp), chymotrypsin (Chy), protease K (PtK), porcine pancreatic elastase (PPE), human neutrophil elastase (HNE), and human neutrophil cathepsin G (CatG). (B) Concentration-dependent inhibition. Inhibition of HNE by serpin-9 (upper) at molar ratios of 0:1, 1.34:1 and 2.68:1 (serpin-9:HNE). Inhibition of bovine trypsin (lower) at molar ratios of 0:1, 0.53:1, 1.06:1, 1.59:1, 2.12:1 (serpin-9:trypsin, open black circle) and 0:1, 0.79:1, 1.05:1, 1.58:1, 2.37:1, 3.16:1, and 3.95:1 (serpin-13:trypsin, closed red circle).

Fig. 5. MALDI-TOF mass spectrometric analysis of the peptides generated in the reactions of serpin-9/11 and serine proteases

A representative single accumulation spectrum is presented for each of the sixteen samples. Protein compositions of the samples are indicated on top of the panels. The mass spectrometer was first calibrated with bovine insulin (5730.61 Da, monoisotopic) and its performance was monitored using the external standard between sample runs. For peaks whose masses match the calculated ones (monoisotopic, *bottom left*), their values are labeled above the peaks: red font for the peptides released upon cleavage between predicted P1 and P1' residues.

Fig. 6. Inhibition of proPO activation in induced plasma (IP, panel A) or 0–50% ammonium sulfate-fractionated plasma (0–50 IP, panel B) by recombinant serpin-9 and -13

One microliter of induced plasma (IP) from the challenged larvae or 0–50 IP was incubated with different amounts of purified recombinant serpin-9 or -13 in 20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM CaCl₂ on ice for 10 min. *M. luteus* (5 μg) was added to each sample to elicit the proPO activation system. After incubation at room temperature for 10 min, PO activities (mean ± SD, n = 3) were measured and plotted along with IP and 0–50 IP alone (negative controls). PO activities IP and 0–50 IP with only *M. luteus* (positive controls) were compared with those in the reactions containing different amounts of serpins by Student's t-test. “*” and “**” indicate statistically significant ($p < 0.05$ and < 0.001) decreases in proPO activation, respectively.

Fig. 7. Immunoblot analysis of the affinity purified hemolymph proteins separated by 10% SDS-PAGE under reducing conditions

The fraction containing serpin-9 (**A**) or serpin-13 (**B**) complexes (10 μ l/lane) was treated with the sample buffer and separated by electrophoresis on a 10% SDS polyacrylamide gel. After electrotransfer, the nitrocellulose membrane was cut into strips which, after blocking, were separately incubated with diluted antisera against the serpins and HPs. Positions and sizes of the M_r standards are indicated on the *left*. The serpin-protease complexes are indicated by arrowheads. Intact serpin-9, -13, and proHP1 are marked by open blue circles. While serpin-13 cleaved at the reactive site loop, as predicted based on its size, is labeled with a blue dot, other bands may represent proteolytic products of the proteins and their complexes.