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Identification of plasma proteinase complexes with serpin-3 in *Manduca sexta*

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Abstract

Extracellular serine proteinase cascades stimulate prophenoloxidase (proPO) activation and antimicrobial peptide production in insect innate immune responses. Serpins in plasma regulate such cascades by selective inhibition of proteinases, in reactions which result in the formation of covalent serpin-proteinase complexes. We carried out experiments to identify plasma proteinases that are inhibited by *Manduca sexta* serpin-3, an immune-inducible serpin known to regulate proPO activation. Immunoaffinity chromatography, using antiserum to serpin-3, yielded serpin-3 complexes with proteinases identified by immunoblot analysis as prophenoloxidase-activating proteinase (PAP)-1, PAP-2, PAP-3, and hemolymph proteinase 8 (HP8). HP8 can cleave and activate the Toll ligand, Spätzle, leading to synthesis of antimicrobial peptides. Analysis by mass spectrometry of tryptic peptides derived from the serpin-3 complexes confirmed the presence of PAP-1, PAP-3, and HP8. Purified recombinant serpin-3 and active HP8 formed an SDS-stable complex *in vitro*. Identification of serpin-3-proteinase complexes in plasma provides insight into proteinase targets of serpin-3 and extends the understanding of serpin/proteinase function in the immune response of *M. sexta*.

Keywords

Serpin; Hemolymph; Immunity; Hemolymph proteinases; Proteomics

1. Introduction

Serpins belong to a superfamily of proteins, many of which inhibit serine proteinases through a suicide mechanism, forming a covalent complex with the proteinase. Serpins consist of a single chain, typically of 350–400 amino acid residues, and share a conserved structure composed of β -sheets and α -helices (Silverman et al., 2001). Between β -sheets A and C, is a flexible region termed the reactive center loop (RCL). The sequence of the RCL determines serpin selectivity. Inhibition of the target proteinase begins with the formation of a noncovalent complex between the serpin and the serine proteinase. However, once the proteinase cleaves the serpin at the P1 residue within the RCL, a covalent ester linkage is formed, which is stable in the presence of SDS. The cleaved serpin undergoes a dramatic

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conformational change, in which the RCL is inserted into β -sheet A, causing a 70 Å translocation and active site distortion of the proteinase (Schulze et al., 1994; Wright, 1996; Silverman et al., 2001; Huntington, 2011).

In insects, serpins in hemolymph have a role in regulating innate immune pathways, including prophenoloxidase activation in *Aedes aegypti* (Zou et al., 2010), *Anopheles gambiae* (Abraham et al., 2005; Michel et al., 2006; An et al., 2011a), *Tenebrio molitor* (Jiang et al., 2009; Park et al., 2011), *Drosophila melanogaster* (Ligoxygakis et al., 2002; De Gregorio et al., 2002; Scherfer et al., 2008; Tang et al., 2008), and *Manduca sexta* (Jiang et al., 2003b; Zhu et al., 2003b; Wang and Jiang, 2004; Tong and Kanost, 2005; Tong et al., 2005; Zou and Jiang, 2005; Wang and Jiang, 2006; An and Kanost, 2010). Regulation of the Toll pathway by serpins has also been observed in *Ae. aegypti* (Shin et al., 2006), *T. molitor* (Jiang et al., 2009; Park et al., 2011), *D. melanogaster* (Levashina et al., 1999; Ahmad et al., 2009), and *M. sexta* (Zou and Jiang, 2005; An and Kanost, 2010; An et al., 2011b).

Seven serpins have been found in *M. sexta* (Kanost, 2007), but endogenous proteinase targets for only a few of the serpins have been identified. Of the twelve serpin-1 alternative splicing isoforms (Jiang et al., 1994; Jiang and Kanost, 1997), proteinase targets for only serpin-1I and serpin-1J have been determined. Serpin-1I inhibits prophenoloxidase activation, and recombinant serpin-1I can form a complex with purified hemolymph proteinase 14 (HP14) (Wang and Jiang, 2006). Serpin-1J can form a complex with prophenoloxidase-activating proteinase-3 (PAP-3) (Jiang et al., 2003b). Serpin-1J also inhibits HP8 to regulate the expression of antimicrobial peptides (An et al., 2011b). Serpin-3 inhibits PAP activity to block prophenoloxidase activation (Zhu et al., 2003b). Serpin-4 and serpin-5 regulate prophenoloxidase activation at steps prior to the PAPs. Serpin-4 inhibits HP21, whereas serpin-5 inhibits HP6 (Tong and Kanost, 2005; Tong et al., 2005). HP6 also stimulates the activation of the Toll pathway, and inhibition of this proteinase by serpin-5 appears to negatively regulate expression of antimicrobial peptide genes (An and Kanost, 2010). Serpin-6 inhibits PAP-3 to regulate prophenoloxidase activation (Wang and Jiang, 2004; Zou and Jiang, 2005) and can form a complex with HP8 (Zou and Jiang, 2005).

Previous studies that identified the PAPs as proteinase targets of serpin-3 (Zhu et al., 2003b) were completed prior to the availability of sequences for many hemolymph proteinases (Jiang et al., 2005; Wang et al., 2006). Therefore, we hypothesize that additional proteinases are also endogenous targets of serpin-3.

In this study, serpin-3 immunoaffinity chromatography was used to purify serpin-3-proteinase complexes from *M. sexta* plasma. Proteinase components identified by immunoblot analysis and analysis of tryptic peptides by mass spectrometry indicated that in addition to the expected serpin-3 complexes with PAPs, a serpin-3-HP8 complex was present in plasma. Inhibitory activity of serpin-3 for HP8 was investigated by using purified recombinant proteins.

2. Materials and Methods

2.1. Insects

Manduca sexta eggs were originally obtained from Carolina Biological Supply and used to establish a laboratory colony. The colony has been maintained by feeding larvae on an artificial diet as previously described by Bell and Joachim (1976).

2.2. Preparation of plasma samples

Fifth-instar, day 3 naïve larvae were chilled on ice for at least 20 min. Hemolymph was collected into individual microcentrifuge tubes by clipping the dorsal horn with scissors. An

equal volume of anti-coagulant saline (4 mM sodium chloride, 40 mM potassium chloride, 8 mM EDTA, 9.5 mM citric acid, 27 mM sodium citrate, 5% sucrose, 0.1 % polyvinylpyrrolidone, and 1.7 mM PIPES, pH 6.5) containing diethyldithiocarbamic acid (final concentration of 2.25 mg/mL) was added to the hemolymph to prevent coagulation and inhibit melanization. Hemocytes were removed by centrifugation at $10,000 \times g$ for 6 min at 4°C. Plasma samples were stored at -80°C.

To obtain plasma from immune-induced larvae with elevated concentration of serpin-3, hemolymph (~30 mL) was collected from thirty-three day 3 fifth-instar larvae 24 h after injection with 100 µg *Micrococcus luteus* (injected as day 2 fifth-instar larvae) into two 50 mL polypropylene centrifuge tubes containing diethyldithiocarbamic acid (final concentration of 2.25 mg/mL) to prevent melanization. Hemocytes were removed by centrifugation at $9000 \times g$ for 15 min at 4°C. The plasma was used for immunoaffinity purification as described in section 2.7.

2.3. Expression and purification of recombinant hemolymph proteinase 8 mutant (HP8-Xa)

A stably transformed *Drosophila* S2 cell line that contains an expression plasmid for *M. sexta* HP8-Xa, a mutant of HP8 that can be activated by bovine factor Xa, has been described previously (An et al., 2009, 2010, 2011b). Six flasks (225 cm²) were seeded with 1.8×10^6 cells/mL in 60 mL of Schneider's *Drosophila* media (Invitrogen) supplemented with heat-inactivated fetal bovine serum (Atlanta Biologicals; final concentration of 10%). After cells were incubated for 3 h at 28°C, HP8-Xa expression was induced by the addition of copper sulfate to a final concentration of 0.5 mM. Medium was collected 48 h after induction by centrifugation at $500 \times g$ for 10 min at 4°C. HP8-Xa secreted into the medium under the control of its own signal peptide was purified by sequential chromatography steps including Affi-gel Blue Gel (150–300 µm; Bio-Rad 153-7301), Concanavalin A Sepharose 4B (GE Healthcare) affinity, Q-Sepharose™ Fast Flow (GE Healthcare) anion exchange, and Sephacryl-S100 High Resolution (GE Healthcare) gel permeation as described by An et al. (2009, 2010).

2.4. Expression and purification of recombinant serpin-3

A cDNA fragment encoding *M. sexta* serpin-3, excluding the signal peptide, was amplified from a previously constructed serpin-3 plasmid (Zhu et al., 2003b) by PCR using a forward primer (S3PETF: 5'-TAC CAT GGG CCA TCA TCA TCA TCA TCA CGG CGA CGA CGT CGA CCC AAA CAC C-3') in which a *Nco*I restriction site (underlined) and sequence encoding a histidine tag (italicized) were introduced and a reverse primer (S3PETR: 5'-ATG CCG CCG CTC AAG CTT TAA AGG CGC CGT C-3') in which a *Not*I restriction site (underlined) was introduced. The PCR product was digested with *Nco*I and *Not*I, analyzed by agarose gel electrophoresis, and visualized using ethidium bromide. A band corresponding to serpin-3 was gel purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the same restriction sites in the pET28a expression vector (Novagen). After sequence verification, the resulting plasmid was used to transform *Escherichia coli* strain BL21 DE3 cells.

A single colony harboring the recombinant plasmid was inoculated into 50 mL of sterile LB containing 50 µL of 50 mg/mL kanamycin in a 250 mL flask and incubated overnight at 37°C with shaking at 250 rpm. Ten mL of the overnight culture were transferred to each of four 2 L flasks containing 500 mL LB and kanamycin (0.5 mL of 50 mg/mL). Flasks were incubated at 37°C with shaking at 250 rpm until the OD₆₀₀ was 0.8 to 1.0. Cultures were transferred to a 20°C incubator with shaking at 150 rpm, and expression of serpin-3 was induced by adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM for 10 h. After storage at 4°C overnight, the bacteria were harvested by centrifugation at $4000 \times g$ for

20 min at 4°C. Pellets were resuspended in cold lysis buffer (300 mM NaCl, 10 mM imidazole in 0.05 M sodium phosphate buffer, pH 8.0) at 4 mL lysis buffer/g cells. Proteinase inhibitor cocktail for His-tagged proteins (Sigma P8849) was also added at 50 µL inhibitor/g cells. Samples were transferred to 50 mL polypropylene centrifuge tubes (Fisher Scientific) and placed in an ice-water bath. Bacteria were sonicated for 10 × 30 seconds with a 1 min rest between bursts using a Vibra Cell Sonics and Materials Sonicator set on level 5 and 50% duty cycle. After sonication, the lysate was centrifuged at 10,000 × g for 30 min at 4°C.

The soluble serpin-3 was first purified by nickel-affinity chromatography (Ni²⁺-NTA). Clear lysate was incubated with Ni²⁺-NTA agarose (Qiagen) at 1 mL agarose for every 4 mL clear lysate in two 50 mL polypropylene centrifuge tubes with rotation for 1 h at 4°C. This mixture was poured into a 1.5 cm diameter column. The column was washed twice with 46 mL of wash buffer (300 mM NaCl, 20 mM imidazole in 0.05 M sodium phosphate buffer, pH 8.0). Recombinant serpin-3 was eluted with 20 mL of elution buffer (300 mM NaCl, 250 mM imidazole in 0.05 M sodium phosphate buffer, pH 8.0). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and Coomassie Blue staining. Fractions containing relatively pure recombinant serpin-3 were pooled and dialyzed at 4°C twice for at least 6 h against 2 L of 20 mM Tris-HCl, pH 8.0 (dialysis buffer), using Slide-A-Lyzer 3.5 K Dialysis Cassettes (~8 mL/cassette; Thermo Scientific). The dialyzed sample was applied to an equilibrated Q-Sepharose™ Fast Flow column (1 × 10 cm) at 1 mL/min. Dialysis buffer was used to wash the column until A₂₈₀ readings were near zero. Proteins bound to the column were eluted with a 60 mL linear gradient of 0–500 mM NaCl in 20 mM Tris-HCl, pH 8.0 at 1 mL/min. Fractions of 1 mL each were collected and analyzed by SDS-PAGE under reducing conditions and Coomassie Blue staining. Fractions containing serpin-3 were pooled and stored at 4°C.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was performed using NuPAGE 4–12% Bis-Tris gels (Invitrogen). Proteins were treated with sample loading buffer containing SDS and β-mercaptoethanol. Samples were heated at 95°C for 5 min and briefly centrifuged. Gels were run in an XCell SureLock™ Electrophoresis Cell (Invitrogen; Novex Mini-Cell) at a constant voltage of 200 V. Gels were stained for 30–60 min in 0.2% (w/v) Coomassie Blue R-250 in 50% (v/v) methanol and 12% (v/v) acetic acid, followed by destaining with 30% (v/v) methanol and 10% (v/v) acetic acid.

For immunoblotting, proteins were transferred to nitrocellulose membranes using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were incubated in 3% dry milk in Tris-buffered saline plus 0.05% Tween-20 with 1:2000 diluted antiserum to *M. sexta* serpin-3 (Zhu et al., 2003b), HP8 (Jiang et al., 2005), and PAPs 1–3 (Wang et al., 2001; Ji et al., 2003, Jiang et al., 2003), followed by incubation with 1:3000 diluted Goat Anti-Rabbit IgG (H+L)-alkaline phosphatase conjugate (Bio-Rad). Blots were developed using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).

2.6. Protein concentration assay

Protein concentrations were determined with the Coomassie Plus™ Protein Assay Reagent (Pierce) using bovine serum albumin (Pierce) as the standard. Alternatively, some samples of purified protein were analyzed by SDS-PAGE together with several bovine serum albumin samples of known amounts, followed by Coomassie Blue staining and estimation of protein concentration by comparison of band intensity with the standards.

2.7. Immunoaffinity purification of serpin-proteinase complexes

Two mL of serpin-3 (KSU 114) or HP8 (OSU 3) antiserum were coupled to Protein-A Sepharose CL-4B (Sigma P3391) according to Harlow and Lane (1988). Serpin-3 or HP8 antibody beads were poured into a 1 cm glass column and washed five times with 5 mL of 100 mM glycine-HCl (pH 2.5) plus 10% ethylene glycol to remove any uncoupled antibody. Antibody-coupled beads were equilibrated with 50 mL of phosphate-buffered saline (PBS), resuspended in 2.5 mL of PBS, and stored at 4°C until use.

Serpin-proteinase complexes were purified from hemolymph according to Tong et al. (2005), with some modification. Plasma (30 mL) from day 3 fifth-instar larvae 24 h after injection with 100 µg *Micrococcus luteus* (see section 2.2) was mixed with phenylthiourea to 1 mM, and *M. luteus* was added to a final concentration of 1 µg/µL to stimulate proteinase activation. This sample was incubated for 30 min at room temperature with rocking. Phenylmethylsulfonyl fluoride (Sigma P-7626; final concentration of 4 mM) and protease inhibitor cocktail (Sigma P8849; 1 mL to 30 mL plasma) were then added to the plasma mixture and incubated for 10 min at room temperature with rocking. The plasma mixture was centrifuged at 5000 × g for 15 min at 4°C to remove debris. The supernatant was added to 1 mL of antibody-coupled beads in 2.5 mL of PBS and rotated horizontally at 4°C overnight. The plasma plus antibody-coupled bead mixture was applied to a 1 cm glass column. Beads were washed with 20 mL of 1 M NaCl followed by 10 mL of 10 mM sodium phosphate buffer, pH 6.5. Bound proteins were eluted with twenty 1 mL aliquots of 100 mM glycine-HCl (pH 2.5), 10% ethylene glycol. Elution fractions were collected into 1.5 microcentrifuge tubes and neutralized with 100 µL of 1 M sodium phosphate buffer, pH 8.0. The fractions were analyzed by SDS-PAGE followed by Coomassie Blue staining and immunoblot analysis.

As a control, Protein-A Sepharose CL-4B (Sigma P3391) beads without antibody-coupling were prepared. Protein-A Sepharose CL-4B beads (0.5 g) were hydrated in ~6 mL of PBS for 2 h at room temperature and then at 4°C overnight. Beads were aliquoted (3 mL) into two 15 mL polypropylene centrifuge tubes (Fisher Scientific). After centrifugation at 1000 × g for 2 min, the pre-swollen beads were washed three times with PBS (10 mL/wash). Approximately 1 mL of beads was resuspended in 3 mL of PBS, poured into a 1 cm glass column, and washed five times with 5 mL of 100 mM glycine-HCl (pH 2.5) plus 10% ethylene glycol. Beads were equilibrated with 50 mL of PBS, resuspended in 2.5 mL of PBS, and stored at 4°C until use. Isolation of proteins from hemolymph that bound to the Protein-A Sepharose CL-4B utilized the same method as described above for the purification of serpin-proteinase complexes. Starting amount of plasma was 12 mL instead of 15 mL, and proteins were eluted with sixteen 1 mL aliquots of 100 mM glycine-HCl (pH 2.5), 10% ethylene glycol. Neutralized elution fractions were subjected to SDS-PAGE followed by Coomassie Blue staining.

2.8. Protein identification by matrix-assisted laser desorption ionization (MALDI) mass spectrometry

Thirty microliters (6.9 µg) of a sample of plasma proteins that bound to a serpin-3 antibody-coupled immunoaffinity column were treated with SDS sample buffer containing β-mercaptoethanol and were subjected to SDS-PAGE using a NuPAGE 4–12% Bis-Tris gel (Invitrogen). The gel was washed three times in deionized water (200 mL/wash) for 5 min, covered with Bio-Safe Coomassie G-250 stain (Bio-Rad 161-0786) for 1 h, and destained with deionized water (200 mL) for 30 min. Bands of interest were excised with a clean razor blade and placed in individual 1.5 mL microcentrifuge tubes containing ~100 µL deionized water. Samples were sent to the Nevada Proteomics Center at the University of Nevada-Reno for analysis.

Spots were digested using a previously described protocol with some modifications (Rosenfeld et al., 1992). Samples were washed twice with 25 mM ammonium bicarbonate and 100% acetonitrile, reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide and incubated with 75 ng sequencing grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate for 6 hours at 37°C. Samples were spotted onto a MALDI target with ZipTip[®]-C18 (Millipore Corp., MA). Samples were eluted with 70% acetonitrile, 0.2% formic acid and overlaid with 0.5 μ l 5 mg/ml MALDI matrix (α -Cyano-4 hydroxycinnamic acid 10 mM ammonium phosphate). All mass spectrometric data were collected using an ABI 4700 Proteomics Analyzer MALDI TOF/TOF mass spectrometer (Applied Biosystems, CA), using their 4000 Series Explorer software v. 3.6. The peptide masses were acquired in reflectron positive mode (1-keV accelerating voltage) from a mass range of 700 – 4000 Daltons, 1250 laser shots were averaged for each mass spectrum. Each sample was internally calibrated on trypsin's autolysis peaks 842.51 and 2211.10 to within 20 ppm. Any sample failing to internally calibrate was analyzed under default plate calibration conditions of 150 ppm. Raw spectrum filtering/peak detection settings were S/N threshold of 3, and cluster area S/N optimization enabled at S/N threshold 10, baseline subtraction enabled at peak width 50. The twenty most intense ions from the MS analysis, which were not on the exclusion list, were subjected to MS/MS. The MS/MS exclusion list included known trypsin masses: 842.51, 870.54, 1045.56, 1126.56, 1420.72, 1531.84, 1940.94, 2003.07, 2211.10, 2225.12, 2239.14, 2283.18, 2299.18, 2678.38, 2807.31, 2914.51, 3094.62, 3337.76, and 3353.75. For MS/MS analysis the mass range was 70 to precursor ion with a precursor window resolution of -1 to +4 Da with an average of 2500 laser shots for each spectrum, CID on, metastable suppressor on. Raw spectrum filtering/peak detection settings were S/N threshold of 5, and cluster area S/N optimization enabled at S/N threshold 6, baseline subtraction enabled at peak width 50. The data was then stored in an Oracle database (Oracle database schema v. 3.19.0 Data version 3.90.0).

The data were extracted from the Oracle database and a peak list was created by GPS Explorer software v 3.6 (Applied Biosystems) from the raw data generated from the ABI 4700. Analyses were performed as combination MS + MS/MS. MS peak filtering included a mass range of 700–4000 Da, minimum S/N filter 10. A peak density filter of 50 peaks per 200 Da with a maximum number of peaks set to 65. MS/MS peak filtering included mass range of 60 Da to 20 Da below each precursor mass. Minimum S/N filter 10, peak density filter of 50 peaks per 200 Da, cluster area filter used with maximum number of peaks 65. The filtered data were searched by Mascot v 1.9.05 (Matrix Science) using NCBI nr database (NCBI 20090915), containing 9,694,989 sequences. Searches were performed without restriction to protein species, M_r , or pI and with variable oxidation of methionine residues and carbamidomethylation of cysteines. Maximum missed cleavage was set to 1 and limited to trypsin cleavage sites. Precursor mass tolerance and fragment mass tolerance were set to 20 ppm and $\pm$ 0.2 Da, respectively.

2.9. Two-dimensional gel electrophoresis

Plasma proteins that bound to a serpin-3 antibody-coupled immunoaffinity column or from the control column of Protein-A Sepharose CL-4B beads without antibody were separated by two-dimensional gel electrophoresis at Kendrick Laboratories, Inc. (Madison, WI). The sample of plasma proteins that bound to a serpin-3 antibody-coupled immunoaffinity column was precipitated with trichloroacetic acid, redissolved in 25 μ L of 5% SDS, 5% β -mercaptoethanol, 10% glycerol and 60 mM Tris, pH 6.8, heated in a boiling water bath for 3 min, and diluted with 25 μ L of 9.5 M urea, 2% (w/v) Nonidet P-40, 5% β -mercaptoethanol, and 2% ampholines consisting of 2% pH 2–11 (Servalytes, Serva, Heidelberg, Germany) before loading. The sample of plasma proteins that bound to a control column of Protein-A

Sepharose CL-4B beads without antibody was treated similarly. For both samples, 46 µg of protein in 50 µL was used for the two-dimensional separation.

Two-dimensional gel electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (O'Farrell, 1975; Burgess-Cassler et al., 1989). Isoelectric focusing (IEF) was carried out in a glass tube of 2.3 mm (inner diameter) using a 2% pH 3.5–10 mix4L ampholines (GE Healthcare, Piscataway, NJ; Serva, Heidelberg, Germany) for 9600 volt-hrs. One microgram of an IEF internal standard, tropomyosin, was added to the sample. Tropomyosin migrates as a doublet and the lower polypeptide spot has a molecular weight of 33,000 and a pI of 5.2.

After equilibration for 10 min in 10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 62.5 mM Tris, pH 6.8, each tube gel was sealed to the top of a stacking gel on top of a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was carried out for about 4 h at 12.5 mA/gel (serpin-3 immunoaffinity sample) or 15 mA/gel (control sample). Myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000) were used as molecular weight standards (Sigma Chemical Co., St. Louis, MO). Gels were stained with Coomassie Blue and shipped in dilute acetic acid.

2.10. Electrospray ionization tandem mass spectrometry

Gel spots excised from the two-dimensional gels were incubated a few times for 10 min in 100 µL of 50% acetonitrile at 30°C, followed by the addition of 50 µL of 100% acetonitrile for 10 min. After discarding the solvent, the gel pieces were dried by speed vacuum concentrator and incubated with 200 ng of sequencing grade trypsin (Trypsin Gold, Promega, Madison, WI) in 20 mM ammonium bicarbonate (total volume of 20 µL). An additional 20 µL of 20 mM ammonium bicarbonate and 10% acetonitrile were added to gel pieces upon rehydration, followed by incubation at 30°C for 17 h. Tryptic peptides were recovered from gel plugs by extraction with 100 µL of 50% acetonitrile in 2% trifluoroacetic acid for 30 min at 30°C. Extracted peptides were concentrated by speed vacuum concentrator and added to 100 µL of 2% acetonitrile in 0.1% formic acid.

Automatic nano-high performance liquid chromatography was performed using a microcolumn switching device (Switchos, LC Packings) coupled to an autosampler (Famos, LC Packings) and a nanogradient generator (UltiMate Nano HPLC, LC Packings). Peptide solution (30 µL) was loaded on a C18 reversed-phase capillary column (75 µm inner diameter × 15 cm; PepMap, Dionex) in conjunction with an Acclaim C18 PepMap trapping column (300 µm inner diameter × 10 mm, Dionex). Peptides were separated by a nanoflow linear acetonitrile gradient using buffer A (0.1% formic acid, 2% acetonitrile) and buffer B (0.1% formic acid, 80% acetonitrile) starting from 5% buffer B to 60% over 45 min at a flow rate of 200 nL/min. Then, the column was washed with 95% buffer B for 5 min. This process was controlled by the system control software, Hystar 3.2. Eluted peptides were injected into an HCT Ultra Ion Trap Mass Spectrometer (Bruker Daltronics). The mass spectrometer was set up in the data dependent MS/MS mode to alternatively acquire full scans (m/z acquisition range from 300 to 1700 Da). The four most intense peaks in any full scan were selected as precursor ions and fragmented by collision energy. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 3.4 and Biotoools 3.0 software (Bruker Daltronics).

Peptide masses were searched by Mascot v 2.3.02 (Matrix Science) using NCBI nr database (NCBI 20110319), containing 13,254,464 sequences. After applying a taxonomy filter to include sequences from *Manduca sexta* only, 772 sequences were searched. Additionally, *M. sexta* ESTs (181,602) from NCBI, a pyrosequencing EST project (Zou et al., 2008; Zhang et

al., 2011), and the *M. sexta* protein database (December 2011), containing 25920 sequences, from Agricultural Pest Genomics Resource Database (www.agripestbase.org) were searched for positive identifications. Searches were performed without restriction to M_r or pI, and with variable oxidation of methionine residues and dehydro of cysteine residues. Maximum missed cleavage was set to 2 and limited to trypsin cleavage sites. The peptide mass tolerance and fragment mass tolerance were set to ± 1.2 Da and ± 0.6 Da, respectively. A threshold of 0.05 was used to determine positive protein identifications and peptides scoring <20 were automatically rejected, ensuring that all protein identifications were based on reliable peptide identifications.

3. Results

3.1. Purification and identification of serpin-3-proteinase complexes in plasma

To investigate serpin-3 as an inhibitor of plasma proteinases, we isolated serpin-3-proteinase complexes from plasma by immunoaffinity chromatography, using serpin-3 antibody. Immunoblot analysis of plasma proteins that bound to the serpin-3 antibody column was carried out using antibodies to serpin-3, HP8, PAP-1, PAP-2, and PAP-3 (Figure 1A). Antibodies to serpin-3 recognized a band at ~ 50 kDa, which is intact serpin-3, and a band at ~ 70 kDa, consistent with the mass expected for a serpin-proteinase complex. Bands between 51 and 64 kDa recognized by the antibody may be cleaved serpin-proteinase complexes, while the band located below 50 kDa probably represents cleaved serpin-3. Bands at the sizes expected for serpin-proteinase complexes were detected in this sample with antibodies to HP8, PAP-1, PAP-2, and PAP-3 (Figure 1A). The zymogen form of HP8 and PAP-1 observed in naïve plasma was not detected in the immunopurified sample. These results suggest that proHP8, proPAP-1, proPAP-2, and proPAP-3 activated in plasma by exposure to bacteria were endogenous targets of serpin-3. Antibodies specific for HP6, HP9, HP10, HP12, HP13, HP14, HP15, HP16, HP17, HP18, HP19, and HP21 were also tested, but no serpin-3-proteinase complexes were detected (data not shown).

PAP-1 and PAP-3 have previously been identified as targets for serpin-3 in *M. sexta* (Zhu et al., 2003b), but HP8 has not. We carried out another immunoaffinity chromatography experiment, using antibodies to HP8, to isolate HP8-serpin complexes in plasma. In immunoblot analysis of the resulting sample (Figure 2), antibodies to HP8 recognized two bands at ~ 70 kDa, consistent with a size expected for a serpin-proteinase complex. This result suggests that HP8 may form a complex with more than one serpin. One putative serpin-proteinase complex was detected with serpin-3 antibody in the sample purified using HP8 antibody. These results further support a conclusion that serpin-3 can form an SDS-stable complex with HP8 in plasma.

To further identify plasma proteins isolated by serpin-3 immunoaffinity, proteins separated by SDS-PAGE (Figure 1B) were digested with trypsin and analyzed by MALDI-TOF/TOF. Resulting peptide masses for each sample were searched against the NCBI nr protein sequence database using Mascot. Protein identifications are listed in Table S1. Serpin-3 (~ 51 kDa) was identified in band 4. Matching peptides were distributed over the entire sequence (covering 65%) and included peptides on the C-terminal side of the P1 Lys residue in the reactive center loop, all consistent with this band representing unmodified serpin-3. Band 2 contained tryptic peptides from PAP-3 and serpin-3. Identified tryptic peptides from PAP-3 covered 19% of the sequence and were located in the catalytic domain of the proteinase (none in the amino-terminal clip domain). This is consistent with the expectation that PAP-3 was activated by cleavage between the clip domain and catalytic domain prior to reacting with serpin-3. Moreover, no tryptic peptides from this band were identified from serpin-3 C-terminal to the P1 Lys residue, consistent with an expectation that serpin-3 was cleaved at this position by PAP-3, resulting in formation of the SDS-stable serpin-proteinase complex.

Band 5, which only contained tryptic peptides from serpin-3, may be cleaved serpin-3. No tryptic peptides C-terminal to the P1 Lys residue were detected in band 5. Serpin-3 was also found in band 3, slightly smaller than the serpin-3-PAP-3 complex. No tryptic peptides for any proteinase were identified in band 3.

Several proteins that were not serpins or proteinases were present in the sample of plasma proteins that were eluted from the serpin-3 antibody column. A band at 200 kDa was apolipoprotein-1 (Figure 1B, band 1) (Kanost et al., 1990; Ryan and van der Horst, 2000). Band 3 contained prophenoloxidase subunit 1 and prophenoloxidase subunit 2. Intense bands (6 and 7) between 28 and 39 kDa were identified as immulectins (Yu and Kanost, 2008). Band 6 contained immulectin-2 and serine proteinase homolog-1. Band 7 included immulectin-3, -4, and -III. Immulectins are pattern recognition proteins that associate with prophenoloxidase and serine proteinase homologs (Yu et al., 2003). Sequences named immulectin-III (AM293329.1) and immulectin-4 (AY768812.2) are 96% identical at the transcript level, suggesting that these two sequences might represent alleles of the same gene. Alternatively, immulectin-III could be encoded by a highly similar gene that arose from gene duplication. Antimicrobial peptides (Kanost et al., 2004; Ragan et al., 2009) were detected in bands 8 and 9, including attacin-1, attacin-II, and gloverin.

To gain higher resolution for protein identification, plasma proteins that bound to the serpin-3 antibody column were separated by two-dimensional gel electrophoresis (Figure 3A) followed by electrospray ionization tandem mass spectrometry of tryptic peptides derived from the resulting protein spots. As a control, plasma proteins eluted from a Protein-A Sepharose CL-4B column lacking antibody were similarly analyzed (Figure 3B). Proteins that bound to the serpin-3 antibody column were identified by using Mascot to search the NCBI protein database restricted to sequences from *M. sexta* (Figure 3A; Table 1; Table S2). Protein scores are a sum of individual ion scores that had a score greater than 22 (significant identity; $p < 0.05$). Only proteins with two or more MS/MS spectra are included in this table. Detailed MS/MS results are listed in Table S7.

Spots 22–26 (Figure 3A; Table S2) are apparently intact serpin-3 and all of these spots contain peptides on the C-terminal side of the P1 Lys residue in the reactive center loop. Since plasma from 15–18 larvae was used for immunoaffinity purification, multiple spots of serpin-3 with small changes in pI are likely due to allelic variation.

Spots 1–5 and 37–39, migrating at ~95 kDa in this gel system, appear to be serpin-3-proteinase complexes (Figure 3A; Table 1). Spot 1 contained serpin-3 and two peptides from the catalytic domain of HP8 (Figure S1). Serpin-3 and PAP-3 were present in spots 2, 3, 4, and 39. No peptides C-terminal to the P1 Lys residue of serpin-3 were detected and only peptides in the catalytic domain of PAP-3 were identified (Figures S2, S3, S4, and S6) in these spots. PAP-1 and serpin-3 were present in spot 5. Like the serpin-3-PAP-3 complexes, no peptides were found after the P1 Lys residue of serpin-3 and only peptides from the catalytic domain of PAP-1 were detected (Figure S5). Percent sequence coverage was calculated by Mascot based on sequences in the NCBI database. In regard to proteinases, this means the zymogen sequence, including the secretion signal peptide. However, proteinases are processed by activation cleavage before interacting with a serpin, and only the catalytic domain should be present in a serpin-proteinase complex. Therefore, the percent sequence coverage for each proteinase in complex was recalculated using the residues in the catalytic domain only (Table 1; values in parentheses). Percent sequence coverage values for the proteinases increased by an average of 4.6% (range of 2.6–6.7%), indicating better sequence coverage by tryptic peptides detected by mass spectrometry. This is to be expected as the catalytic domain contains fewer amino acids than the zymogen sequence from NCBI. Spots 37 and 38 have a molecular weight consistent with a serpin-

proteinase complex. They clearly contained serpin-3, but no proteinase was identified. These may represent complexes of serpin-3 with proteinases in plasma that have not yet been discovered.

Spots 15–19, 43, and 52 all contained serpin-3 (Figure 3A; Table S2), and are at molecular weights that are consistent with cleaved serpin-proteinase complexes. In addition to serpin-3, spot 17 contained seven peptides from a protein similar to a gram-negative binding protein (Wang et al., 2011).

Spots 27–30 were smaller than intact serpin-3, but contained serpin-3 peptides (Figure 3A; Table S2). These spots, with the exception of 27, did not contain peptides C-terminal to the P1 Lys residue, suggesting that they were cleaved in the RCL, but reacted as proteinase substrate rather than inhibitor.

Other immune-related proteins were also identified in the plasma proteins that bound to the serpin-3 antibody column. Immulectins (2, 2a, 3, III, and 4) were found in spots 3, 4, 13, 15, 67, 59, 60, 61 (Table S2), and in a series of very intensely stained spots 54–58 (Table 2). Serine proteinase homologs (1b, 2, and 4), which are necessary for prophenoloxidase activation, were found in spots 20–22, 27–30, 33–35, and 56. Prophenoloxidase subunit 1 was found in spots 7–9, and prophenoloxidase subunit 2 was found in spots 6, 10–14, 30, and 51. Attacin-1, an antimicrobial peptide, was in spots 66–68. HP14, known to initiate a prophenoloxidase pathway, was identified in spot 41.

To find additional proteins that do not have sequences in the NCBI protein database, the ESI-MS/MS data was searched against *M. sexta* expressed sequence tags (ESTs) compiled from NCBI and a new pyrosequencing EST project (Zou et al., 2008; Zhang et al., 2011), and the *M. sexta* protein database (December 2011) from Agricultural Pest Genomics Resource Database (www.agripestbase.org). Table S3 lists the top Mascot hits obtained from searching the ESTs, and protein scores composed of individual ion scores that had a score greater than 46 were considered to have significant identity ($p < 0.05$). Identification of ESTs based on BLAST searches against the NCBI database and proteins from the *M. sexta* protein database (data not shown) were consistent with results obtained by searching the NCBI protein database restricted to *M. sexta*.

To investigate the potential for plasma proteins to interact nonspecifically with the affinity column, a control experiment was carried out using a column of Protein-A Sepharose CL-4B beads with no coupled antibody. Plasma proteins that bound to the control column were identified by using Mascot to search the NCBI protein database restricted to sequences from *M. sexta* (Figure 3B; Table 2; Table S4). These ESI-MS/MS data were also searched against *M. sexta* expressed sequence tags compiled from NCBI and a new pyrosequencing EST project (Zou et al., 2008; Zhang et al., 2011) to find additional proteins that do not have sequences in the NCBI protein database (Table S5). Detailed MS/MS data are included in Table S8.

Immulectins (2b, 3, III, and 4) were very prominent proteins that bound to the Protein-A Sepharose CL-4B beads lacking antibody (Figure 3B; Table 2; Table S4). Spots containing immulectins included 1c-6c, 12c-21c, and 23c-30c. Identification of immulectins in spots 12c-14c are most likely a separation artifact as their location is not consistent with the expected pI of immulectin-2b (pI = 5.76) or immulectin-3 (pI = 5.86). However, spot 13c also contained immulectin-III, which has a calculated pI of 9.23. Prophenoloxidase subunit 2 was found in spot 11c. Serine proteinase homologs were present in spots 22c (SPH-2) and 28c (SPH-1b).

No spots for serpin-3 or for serine proteinases were identified from the control column, indicating that serpin-3-proteinase complexes isolated using the antibody column were due to specific interaction with the serpin-3 antibodies. However, the very abundant spots containing several immunoglobulins and serine proteinase homologs from both the immunoaffinity column and the control column are likely to have resulted from nonspecific interactions of these proteins with the column matrix.

3.2. Complex formation between purified, recombinant HP8 and serpin-3

To further investigate the interaction between HP8 and serpin-3, we prepared a recombinant form of HP8 mutated to permit its activation by bovine factor Xa as previously described (An et al., 2009, 2010, 2011b). After recombinant serpin-3 was mixed with activated HP8-Xa, a higher molecular weight complex (~70 kDa) was detected by immunoblot analysis using antiserum to serpin-3 or HP8 (Figure 4). In contrast, when serpin-3 was mixed with proHP8-Xa zymogen, anti-HP8 antibodies recognized only the 42 kDa proHP8-Xa zymogen and a 37 kDa truncated form of HP8. After incubation with factor Xa, the proHP8-Xa zymogen disappeared, and the 34 kDa HP8 catalytic domain was detected. Mixing serpin-3 with active HP8-Xa resulted in decreased intensity of the 34 kDa catalytic domain and the appearance of a new immunoreactive band at 70 kDa, consistent with the expected size for a serpin-proteinase complex. This serpin-proteinase complex band was absent in lanes for various negative controls, and HP8 antibody does not recognize bovine factor Xa (An et al., 2010). These results using purified recombinant proteins confirm that serpin-3 can form a covalent complex with active HP8, as typically observed for serpin-protease inhibition reactions.

4. Discussion

A previous study to identify proteinase targets of serpin-3 (Zhu et al., 2003b) was completed prior to the discovery of sequences for many hemolymph proteinases in *M. sexta* (Jiang et al., 2005; Wang et al., 2006). PAP-1 and PAP-3 were identified as targets of serpin-3 by immunoaffinity chromatography followed by immunoblot analysis (Zhu et al., 2003b). In the current study, we re-examined serpin-3 and its proteinase targets by purifying serpin-3-proteinase complexes from plasma activated by treatment with bacteria, using a serpin-3 immunoaffinity column. Proteinase components were identified by immunoblot analysis and analysis of tryptic peptides by MALDI-TOF/TOF or ESI-MS/MS after separation by one- or two-dimensional SDS-PAGE. Both of these strategies have been utilized to identify proteinase targets of other *M. sexta* serpins (Tong et al., 2005; Ragan et al., 2010). One-dimensional SDS-PAGE followed by immunoblot and MALDI-TOF analysis was used to identify serpin-4 or serpin-5-proteinase complexes in plasma. Serpin-4 formed a complex with HP1, HP6, and HP21, whereas serpin-5 formed a complex with HP1 and HP6 (Tong et al., 2005). Two-dimensional SDS-PAGE followed by immunoblot and MALDI-TOF/TOF analysis identified serpin-1A, -1E, and -1J in complex with HP8, and an unidentified serpin-1 isoform formed a complex with HP1 (Ragan et al., 2010).

Results in this study demonstrated that serpin-3 formed complexes in plasma with at least three proteinases, HP8, PAP-1, and PAP-3, which are all clip domain serine proteinases with at least one amino-terminal clip domain and a C-terminal serine proteinase domain (Jiang et al., 2005). These proteinases have trypsin-like specificity (Jiang et al., 1998, 2003b, 2005; An et al., 2009, 2010), consistent with the cleavage at the Lys P1 residue in the RCL of serpin-3 (Zhu et al., 2003b). The existence in plasma of these proteinases in complexes with serpin-3 was confirmed by immunoblot analysis using antibodies to the proteinases and to serpin-3. Identification of serpin-3-PAP-1 and serpin-3-PAP-3 complexes by immunoblot analysis is consistent with results obtained by Zhu et al. (2003b). In the current study, one-dimensional SDS-PAGE followed by trypsin digestion and MALDI-TOF/TOF analysis of

selected bands identified tryptic peptides of both serpin-3 and PAP-3 in the same band; however, tryptic peptides for HP8, PAP-1, or PAP-2 were not identified by this method. To achieve better separation, two-dimensional SDS-PAGE was performed, and proteins were identified by ESI-MS/MS analysis. Eight spots (1–5, 37–39) were consistent with the size of a serpin-3 proteinase complex. Two to five tryptic peptides for the catalytic domain of PAP-3 were found in spots 2, 3, 4, and 39 along with serpin-3. A total of six tryptic peptides for PAP-1 were found with serpin-3 in spot 5. Spot 1 contained serpin-3 and two tryptic peptides from the catalytic domain of HP8. Although immunoblot analysis detected a putative serpin-3-PAP-2 complex in plasma, no tryptic peptides from PAP-2 were detected by mass spectrometry. It is possible that PAP-2 antiserum may have detected a different protease complex due to cross-reaction with another hemolymph protease, which may be PAP-3 (Jiang et al. 2003a). Spots 37 and 38 contained serpin-3, but no proteinase components were identified. These may represent proteinases in plasma that have not yet been identified. We conclude that PAP-1, PAP-3, and HP8 appear to be the major targets for inhibition by serpin-3 *in vivo*.

Immunelectins, prophenoloxidase subunits 1 and 2, and serine proteinase homologs bound to the serpin-3 immunoaffinity column. These proteins are all known components of the prophenoloxidase activation pathway (Kanost et al., 2004). This result is consistent with immunoaffinity purification of serpin-4 or -5-proteinase complexes (Tong et al., 2005), in which large amounts of immunelectins and serine proteinases homologs co-purified with serpin-proteinase complexes. Tong et al. (2005) speculated that immunelectin-2 might recognize carbohydrates on the surface of bacteria (Yu and Kanost, 2000) and function as a linker or platform to localize components of the prophenoloxidase activation system at a microbial surface. Serine proteinase homologs bind to immunelectin-2 and prophenoloxidase (Yu et al., 2003) suggesting that the formation of proteinase complexes, including proteinases inhibited by serpins, are held together by their interaction with serine proteinase homologs and immunelectins (Tong et al., 2005).

We carried out a control experiment, using a column of Protein-A Sepharose CL-4B beads without antibody, to determine whether some plasma proteins might bind to the column in a manner not depending on serpin-3 antibody. We identified immunelectin-2 as a very prominent protein in the protein fraction that bound to the column, with lesser amounts of other immunelectins, serine proteinase homologs 1 and 2, and prophenoloxidase. These results are consistent with a hypothesis that immunelectins bind to carbohydrates present on the Protein-A Sepharose CL-4B beads and that serine proteinase homologs and prophenoloxidase interact with bound immunelectins. Furthermore, this control experiment indicates that the binding of those components to the column is independent of interaction with serpins of serpin-proteinase complexes, which were not present in the control fractions, in contrast to our previous speculation (Tong et al., 2005) that serpin-proteinase complexes were interacting with a larger, immunelectin-mediated complex.

Several other proteins identified in the plasma proteins that bound to the serpin-3 immunoaffinity column have potential immune functions. These included a gram-negative binding protein, named microbe binding protein, (Wang et al., 2011), HP14, a modular serine proteinase that autoactivates in the presence of β -1,3-glucan and β GRP2 to stimulate the activation of prophenoloxidase (Wang and Jiang, 2006), antimicrobial proteins, including attacin-1, attacin-II, and gloverin (Engström et al., 1984; Carlsson et al., 1991; Axén et al., 1997), inter- α -trypsin inhibitor heavy chain (Zhu et al., 2003a), and a Kazal family serine proteinase inhibitor (Zhu et al., 2003a).

We tested the interaction between purified, recombinant HP8 and serpin-3. Formation of an SDS-stable complex, a hallmark feature of a serpin-proteinase interaction, was tested by

incubating recombinant serpin-3 with a form of HP8 activated by bovine factor Xa (HP8-Xa). We determined that serpin-3 and HP8 form an SDS-stable complex *in vitro*. Samples containing factor Xa alone did not contain a band at the size of the complex, indicating that the observed complex was due to the interaction of serpin-3 with active HP8-Xa and not factor Xa. Similar *in vitro* complexes have been detected for serpin-1J with PAP-3 or HP8 (Jiang et al., 2003b; An et al., 2011b), serpin-3 with PAP-1 or PAP-3 (Zhu et al., 2003b), serpins-4 and -5 with PAP-1 or HP6 (Tong and Kanost, 2005; An and Kanost., 2010), serpin-1I with HP14 (Wang and Jiang, 2006), serpin-6 with PAP-3 (Zou and Jiang, 2005), and serpin-4 with HP21 (Wang and Jiang, 2007). This result further supports the hypothesis that serpin-3 may be a biologically relevant inhibitor of HP8, which stimulates the production of antimicrobial peptides by cleaving and activating the Toll ligand, Spätzle (An et al., 2009, 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HP	hemolymph proteinase
PAP	prophenoloxidase-activating proteinase
proPO	prophenoloxidase
SPH	serine proteinase homolog
MALDI-TOF/TOF	matrix-assisted laser desorption ionization-time of flight tandem mass spectrometry
ESI-MS/MS	electrospray ionization tandem mass spectrometry
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
serpin	serine proteinase inhibitor
RCL	reactive center loop
EST	expressed sequence tag
IEF	isoelectric focusing

References

- Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M, Michel K. An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc Natl Acad Sci USA*. 2005; 102:16327–16332. [PubMed: 16260729]

- Ahmad ST, Sweeney ST, Lee JA, Sweeney NT, Gao FB. Genetic screen identifies serpin5 as a regulator of the toll pathway and CHMP2B toxicity associated with frontotemporal dementia. *Proc Natl Acad Sci USA*. 2009; 106:12168–12173. [PubMed: 19581577]
- An C, Budd A, Kanost MR, Michel K. Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. *Cell Mol Life Sci*. 2011a; 68:1929–1939. [PubMed: 20953892]
- An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR. Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. *J Biol Chem*. 2009; 284:19716–19726. [PubMed: 19487692]
- An C, Jiang H, Kanost MR. Proteolytic activation and function of the cytokine Spätzle in the innate immune response of a lepidopteran insect, *Manduca sexta*. *FEBS J*. 2010; 277:148–162. [PubMed: 19968713]
- An C, Kanost MR. *Manduca sexta* serpin-5 regulates prophenoloxidase activation and the Toll signaling pathway by inhibiting hemolymph proteinase HP6. *Insect Biochem Mol Biol*. 2010; 40:683–689. [PubMed: 20624461]
- An C, Ragan EJ, Kanost MR. Serpin-I splicing isoform J inhibits the proSpätzle-activating proteinase HP8 to regulate expression of antimicrobial hemolymph proteins in *Manduca sexta*. *Dev Comp Immunol*. 2011b; 35:135–141. [PubMed: 20851714]
- Axén A, Carlsson A, Engström A, Bennich H. Gloverin, an antibacterial protein from the immune hemolymph of *Hyalophora* pupae. *Eur J Biochem*. 1997; 247:614–619. [PubMed: 9266704]
- Bell RA, Joachim FG. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann Entomol Soc Amer*. 1976; 69:365–373.
- Burgess-Cassler A, Johansen JJ, Santek DA, Ide JR, Kendrick NC. Computerized quantitative analysis of coomassie-blue-stained serum proteins separated by two-dimensional electrophoresis. *Clin Chem*. 1989; 35:2297–2304. [PubMed: 2480196]
- Carlsson A, Engström P, Palva ET, Bennich H. Attacin, an antibacterial protein from *Hyalophora cecropia*, inhibits synthesis of outer membrane proteins in *Escherichia coli* by interfering with *omp* gene transcription. *Infect Immun*. 1991; 59:3040–3045. [PubMed: 1715318]
- De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata S, Lee BL, Iwanaga S, Lemaitre B, Brey PT. An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. *Dev Cell*. 2002; 3:581–592. [PubMed: 12408809]
- Engström P, Carlsson A, Engström A, Tao ZJ, Bennich H. The antibacterial effect of attacins from the silk moth *Hyalophora cecropia* is directed against the outer membrane of *Escherichia coli*. *EMBO J*. 1984; 3:3347–3351. [PubMed: 6396089]
- Harlow, E.; Lane, D. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1988.
- Huntington JA. Serpin structure, function and dysfunction. *J Thromb Haemost*. 2011; 9(Suppl 1):26–34. [PubMed: 21781239]
- Ji C, Wang Y, Ross J, Jiang H. Expression and in vitro activation of *Manduca sexta* prophenoloxidase-activating proteinase-2 precursor (proPAP-2) from baculovirus-infected insect cells. *Protein Expr Purif*. 2003; 29:235–243. [PubMed: 12767815]
- Jiang H, Kanost MR. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-I from *Manduca sexta*. *J Biol Chem*. 1997; 272:1082–1087. [PubMed: 8995406]
- Jiang H, Wang Y, Gu Y, Guo X, Zou Z, Scholz F, Trenczek TE, Kanost MR. Molecular identification of a bevy of serine proteinases in *Manduca sexta* hemolymph. *Insect Biochem Mol Biol*. 2005; 35:931–943. [PubMed: 15944088]
- Jiang H, Wang Y, Kanost MR. Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila* easter. *Proc Natl Acad Sci USA*. 1998; 95:12220–12225. [PubMed: 9770467]
- Jiang H, Wang Y, Kanost MR. Mutually exclusive exon use and reactive center diversity in insect serpins. *J Biol Chem*. 1994; 269:55–58. [PubMed: 8276850]
- Jiang H, Wang Y, Yu XQ, Kanost MR. Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*: A bacteria-inducible serine proteinase containing two clip domains. *J Biol Chem*. 2003a; 278:3552–3561. [PubMed: 12456683]

- Jiang H, Wang Y, Yu XQ, Zhu Y, Kanost MR. Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem Mol Biol*. 2003b; 33:1049–1060. [PubMed: 14505699]
- Jiang R, Kim EH, Gong JH, Kwon HM, Kim CH, Ryu KH, Park JW, Kurokawa K, Zhang J, Gubb D, Lee BL. Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. *J Biol Chem*. 2009; 284:35652–35658. [PubMed: 19858208]
- Kanost, MR. Serpins in a lepidopteran insect, *Manduca sexta*. In: Silverman, GA.; Lomas, DA., editors. *The Serpinopathies: Molecular and Cellular Aspects of Serpins and their Disorders*. World Scientific Publishing Co; Hackensack, NJ: 2007. p. 229-242.
- Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol Rev*. 2004; 198:97–105. [PubMed: 15199957]
- Kanost, MR.; Kawooya, JK.; Law, JH.; Ryan, RO.; Van Heusden, MC.; Ziegler, R. Insect haemolymph proteins. In: Evans, PD.; Wigglesworth, VB., editors. *Advances in Insect Physiology*. Academic Press; San Diego: 1990. p. 299-396.
- Levashina EA, Langley E, Green C, Gubb D, Ashburner M, Hoffmann JA, Reichhart JM. Constitutive activation of toll-mediated antifungal defense in serpin-deficient *Drosophila*. *Science*. 1999; 285:1917–1919. [PubMed: 10489372]
- Ligoxygakis P, Pelte N, Ji C, Leclerc V, Duvic B, Belvin M, Jiang H, Hoffmann JA, Reichhart JM. A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*. *EMBO J*. 2002; 21:6330–6337. [PubMed: 12456640]
- Michel K, Suwanchaichinda C, Morlais I, Lambrechts L, Cohuet A, Awono-Ambene PH, Simard F, Fontenille D, Kanost MR, Kafatos FC. Increased melanizing activity in *Anopheles gambiae* does not affect development of *Plasmodium falciparum*. *Proc Natl Acad Sci USA*. 2006; 103:16858–16863. [PubMed: 17065316]
- O'Farrell PH. High resolution two-dimensional electrophoresis of proteins. *J Biol Chem*. 1975; 250:4007–4021. [PubMed: 236308]
- Park SH, Jiang R, Piao S, Zhang B, Kim EH, Kwon HM, Jin XL, Lee BL, Ha NC. Structural and functional characterization of a highly specific serpin in the insect innate immunity. *J Biol Chem*. 2011; 286:1567–1575. [PubMed: 21047786]
- Ragan, EJ.; An, C.; Jiang, H.; Kanost, MR. Roles of haemolymph proteins in antimicrobial defences of *Manduca sexta*. In: Rolff, J.; Reynolds, SE., editors. *Insect Infection and Immunity*. Oxford University Press; 2009. p. 34-48.
- Ragan EJ, An C, Yang CT, Kanost MR. Analysis of mutually exclusive alternatively spliced serpin-1 isoforms and identification of serpin-1 proteinase complexes in *Manduca sexta* hemolymph. *J Biol Chem*. 2010; 285:29642–29650. [PubMed: 20624920]
- Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem*. 1992; 203:173–179. [PubMed: 1524213]
- Ryan RO, van der Horst DJ. Lipid transport biochemistry and its role in energy production. *Annu Rev Entomol*. 2000; 45:233–260. [PubMed: 10761577]
- Scherfer C, Tang H, Kambris Z, Lhocine N, Hashimoto C, Lemaitre B. *Drosophila* Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. *Dev Biol*. 2008; 323:189–196. [PubMed: 18801354]
- Schulze AJ, Huber R, Bode W, Engh RA. Structural aspects of serpin inhibition. *FEBS Lett*. 1994; 344:117–124. [PubMed: 8187869]
- Shin SW, Bian G, Raikhel AS. A toll receptor and a cytokine, Toll5A and Spz1C, are involved in toll antifungal immune signaling in the mosquito *Aedes aegypti*. *J Biol Chem*. 2006; 281:39388–39395. [PubMed: 17068331]
- Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PG, Irving JA, Lomas DA, Luke CJ, Moyer RW, Pemberton PA, Remold-O'Donnell E, Salvesen GS, Travis J, Whisstock JC. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J Biol Chem*. 2001; 276:33293–33296. [PubMed: 11435447]

- Tang H, Kambris Z, Lemaitre B, Hashimoto C. A serpin that regulates immune melanization in the respiratory system of *Drosophila*. *Dev Cell*. 2008; 15:617–626. [PubMed: 18854145]
- Tong Y, Jiang H, Kanost MR. Identification of plasma proteases inhibited by *Manduca sexta* serpin-4 and serpin-5 and their association with components of the prophenol oxidase activation pathway. *J Biol Chem*. 2005; 280:14932–14942. [PubMed: 15695806]
- Tong Y, Kanost MR. *Manduca sexta* serpin-4 and serpin-5 inhibit the prophenol oxidase activation pathway: cDNA cloning, protein expression, and characterization. *J Biol Chem*. 2005; 280:14923–14931. [PubMed: 15695807]
- Wang Y, Jiang H. Reconstitution of a branch of the *Manduca sexta* prophenoloxidase activation cascade in vitro: snake-like hemolymph proteinase 21 (HP21) cleaved by HP14 activates prophenoloxidase-activating proteinase-2 precursor. *Insect Biochem Mol Biol*. 2007; 37:1015–1025. [PubMed: 17785189]
- Wang Y, Jiang H. Interaction of beta-1,3-glucan with its recognition protein activates hemolymph proteinase 14, an initiation enzyme of the prophenoloxidase activation system in *Manduca sexta*. *J Biol Chem*. 2006; 281:9271–9278. [PubMed: 16461344]
- Wang Y, Jiang H. Purification and characterization of *Manduca sexta* serpin-6: a serine proteinase inhibitor that selectively inhibits prophenoloxidase-activating proteinase-3. *Insect Biochem Mol Biol*. 2004; 34:387–395. [PubMed: 15041022]
- Wang Y, Jiang H, Kanost MR. Expression and purification of *Manduca sexta* prophenoloxidase-activating proteinase precursor (proPAP) from baculovirus-infected insect cells. *Protein Expr Purif*. 2001; 23:328–337. [PubMed: 11676609]
- Wang Y, Sumathipala N, Rayaprolu S, Jiang H. Recognition of microbial molecular patterns and stimulation of prophenoloxidase activation by a beta-1,3-glucanase-related protein in *Manduca sexta* larval plasma. *Insect Biochem Mol Biol*. 2011; 41:322–331. [PubMed: 21296155]
- Wang Y, Zou Z, Jiang H. An expansion of the dual clip-domain serine proteinase family in *Manduca sexta*: gene organization, expression, and evolution of prophenoloxidase-activating proteinase-2, hemolymph proteinase 12, and other related proteinases. *Genomics*. 2006; 87:399–409. [PubMed: 16324822]
- Wright HT. The structural puzzle of how serpin serine proteinase inhibitors work. *Bioessays*. 1996; 18:453–464. [PubMed: 8787534]
- Yu XQ, Jiang H, Wang Y, Kanost MR. Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem Mol Biol*. 2003; 33:197–208. [PubMed: 12535678]
- Yu, X.; Kanost, MR. Activation of lepidopteran insect innate immune responses by C-type immulectins. In: Ahmed, HA.; Vasta, GR., editors. *Animal Lectins: A Functional View*. CRC Press; Boca Raton: 2008. p. 383-395.
- Yu XQ, Kanost MR. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *Manduca sexta*, is induced in response to gram-negative bacteria. *J Biol Chem*. 2000; 275:37373–37381. [PubMed: 10954704]
- Zhang S, Gunaratna RT, Zhang X, Najjar F, Wang Y, Roe B, Jiang H. Pyrosequencing-based expression profiling and identification of differentially regulated genes from *Manduca sexta*, a lepidopteran model insect. *Insect Biochem Mol Biol*. 2011; 41:733–746. [PubMed: 21641996]
- Zhu Y, Johnson TJ, Myers AA, Kanost MR. Identification by subtractive suppression hybridization of bacteria-induced genes expressed in *Manduca sexta* fat body. *Insect Biochem Mol Biol*. 2003a; 33:541–559. [PubMed: 12706633]
- Zhu Y, Wang Y, Gorman MJ, Jiang H, Kanost MR. *Manduca sexta* serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases. *J Biol Chem*. 2003b; 278:46556–46564. [PubMed: 12966082]
- Zou Z, Jiang H. *Manduca sexta* serpin-6 regulates immune serine proteinases PAP-3 and HP8. cDNA cloning, protein expression, inhibition kinetics, and function elucidation. *J Biol Chem*. 2005; 280:14341–14348. [PubMed: 15691825]
- Zou Z, Najjar F, Wang Y, Roe B, Jiang H. Pyrosequence analysis of expressed sequence tags for *Manduca sexta* hemolymph proteins involved in immune responses. *Insect Biochem Mol Biol*. 2008; 38:677–682. [PubMed: 18510979]

Zou Z, Shin SW, Alvarez KS, Kokoza V, Raikhel AS. Distinct melanization pathways in the mosquito *Aedes aegypti*. *Immunity*. 2010; 32:41–53. [PubMed: 20152169]

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Highlights

1. Serpin-3-proteinase complexes were isolated by immunoaffinity chromatography.
2. HP8, PAP-1, and PAP-3 were identified in complex with serpin-3 by immunoblot and proteomic analysis.
3. Active HP8 formed a SDS-stable complex with recombinant serpin-3 *in vitro*.

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Figure 1. Detection of complexes between serpin-3 and HP8, PAP-1, PAP-2, and PAP-3 in plasma

Fifth-instar, day 3 naïve plasma (lane 1) and a sample of plasma proteins that bound to a serpin-3 antibody affinity column (lane 2) were subjected to SDS-PAGE under reducing conditions followed by immunoblot analysis with antiserum to serpin-3, HP8, PAP-1, PAP-2, or PAP-3 (A) and Coomassie Blue staining (B). Coomassie Blue stained bands were excised for trypsin digestion followed by MALDI-TOF and MALDI-TOF/TOF mass spectrometry analysis. Numbers in parentheses correspond to the band number in Table S1.

Figure 2. Immunoaffinity separation using antibody to HP8 confirms association of serpin-3 with HP8

Naïve plasma (lane 1) and a sample of plasma proteins that bound to a HP8 antibody affinity column (lane 2) were subjected to SDS-PAGE under reducing conditions followed by immunoblot analysis with antiserum to HP8 or serpin-3.

Figure 3. Two-dimensional gel electrophoresis analysis of plasma proteins isolated by serpin-3 antibody immunoaffinity

A sample of plasma proteins that bound to a serpin-3 antibody-coupled immunoaffinity column (A) and a control column of Protein-A Sepharose CL-4B beads without antibody (B) was separated by two-dimensional gel electrophoresis, using a pH range of 3.5–10 for isoelectric focusing (tropomyosin internal standard, pI = 5.2) and a 10% acrylamide slab gel for molecular size separation. Numbers indicate Coomassie Blue stained spots that were subjected to trypsin digestion followed by ESI-MS/MS mass spectrometry (Tables 1 and 2; Tables S2, S3, S4, and S5).

Figure 4. Recombinant HP8-Xa and serpin-3 form an SDS-stable complex *in vitro*

Recombinant proHP8-Xa (50 ng=1.3 pmol) was incubated with bovine factor Xa (100 ng) in 20 mM Tris-HCl, 150 mM NaCl, 0.005% Tween-20, pH 8.0, for 23.5 h at 37°C to produce active HP8. Recombinant serpin-3 (636.3 ng=13 pmol) was added to the activated mixture and incubated for 10 min at room temperature. Mixtures were subjected to SDS-PAGE under reducing conditions followed by immunoblot analysis with antiserum to serpin-3 or HP8.

Table 1

Identification of serpin-3 and serpin-3-proteinase complexes from 2D gel analysis of serpin-3 immunoaffinity purified hemolymph proteins.

Spot	Protein name	GenInfo identifier	Protein MW	Protein score ^a	Peptide count	% sequence coverage ^b
1	serpin 3a	gi 27733415	51096	1311	19	45.7
	HP8	gi 56418397	40710	122	2	6.7 (9.7)
2	serpin 3a	gi 27733415	51096	1390	22	54.1
	PAP-3	gi 35277829	46240	119	2	4.7 (7.3)
3	serpin 3a	gi 27733415	51096	1459	22	52.5
	PAP-3	gi 35277829	46240	180	4	9.6 (15.3)
4	serpin 3a	gi 27733415	51096	1462	23	52.7
	PAP-3	gi 35277829	46240	200	5	10.5 (17.2)
5	serpin 3a	gi 27733415	51096	1495	23	56
	PAP-1	gi 60299968	42785	356	6	13.3 (19.9)
37	serpin 3a	gi 27733415	51096	935	16	33.4
38	serpin 3a	gi 27733415	51096	1291	21	46.2
39	serpin 3a	gi 27733415	51096	873	15	28.8
	PAP-3	gi 35277829	46240	79	2	4.4 (7.3)

^aSum of individual ion scores excluding duplicate matches. Scores > 22 indicate significant identity ($p < 0.05$).

^bPercent coverage values in parentheses are calculated by using the residues in the catalytic domain of the proteinase only.

Table 2

Immune-related proteins identified from 2D gel analysis of serpin-3 immunoaffinity purified hemolymph proteins.

Spot ^d	Protein name	GenInfo identifier	Protein MW	Protein score ^b	Peptide count	% sequence coverage
55	immulectin-2b	gi 224381231	37069	452	7	27.8
56	immulectin-2b	gi 224381231	37069	211	5	19.6
	immulectin-2a	gi 224381229	37069	211	5	19.6
	SPH-1b	gi 242351233	45594	204	4	7.0
	immulectin III	gi 110649250	34789	80	2	4.6
57	immulectin-4	gi 237861314	35559	279	5	15.4
	immulectin III	gi 110649250	34789	277	4	11.1
	immulectin-2b	gi 224381231	37069	114	3	10.4
	dVA-AP7	gi 110649220	16486	106	2	14.0
58	immulectin-3	gi 55139125	33846	139	3	10.6
27c	immulectin-2b	gi 224381231	37069	424	7	27.8
28c	immulectin-2b	gi 224381231	37069	250	5	17.4
	SPH-1b	gi 242351233	45594	99	3	10.3
29c	immulectin III	gi 110649250	34789	219	4	11.1
	immulectin-4	gi 237861314	35559	201	4	11.0
	immulectin-3	gi 55139125	33846	27	1	3.9
30c	immulectin-3	gi 55139125	33846	69	2	6.1

^aSpots identified with a number followed by the letter "c" indicate proteins that bound a control column of Protein-A Sepharose CL-4B beads without antibody attached.

^bSum of individual ion scores excluding duplicate matches. Scores > 22 indicate significant identity ($p < 0.05$).