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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*.



## 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 conformational change, in which the RCL is inserted into β-sheet A, causing a 70Å 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.,

*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.

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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

*M. 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% polyvinylpyrollidone, 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 × 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 × 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 $cm^2$) 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 × 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 C*CA TCA 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 CGG 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 $OD_{600}$ was 0.8–1.0. 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 × 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 s 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 ($Ni^{2+}$-NTA). Clear lysate was incubated with $Ni^{2+}$-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