**Manduca sexta** Serpin-6 Regulates Immune Serine Proteinases PAP-3 and HP8

cDNA CLONING, PROTEIN EXPRESSION, INHIBITION KINETICS, AND FUNCTION ELUCIDATION*

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Analogous to blood coagulation and complement activation in mammals, some insect defense responses (*e.g.* prophenoloxidase (proPO) activation and Toll pathway initiation) are mediated by serine proteinase cascades and regulated by serpins in hemolymph. We recently isolated *Manduca sexta* serpin-6 from hemolymph of the bacteria-challenged larvae, which selectively inhibited proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *M. sexta* serpin-6 is 55% similar in amino acid sequence to *Drosophila melanogaster* proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *M. sexta* serpin-6 is 55% similar in amino acid sequence to *Drosophila melanogaster* proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *M. sexta* serpin-6 is 55% similar in amino acid sequence to *Drosophila melanogaster* proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *M. sexta* serpin-6 is 55% similar in amino acid sequence to *Drosophila melanogaster* proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *M. sexta* serpin-6 is 55% similar in amino acid sequence to *Drosophila melanogaster* proPO-activating proteinase-3 (PAP-3) (Wang, Y., and Jiang, H. (2004) *Insect Biochem. Mol. Biol.* 34, 387–395). To further characterize its structure and function, we cloned serpin-6 from an induced fat body cDNA library using a PCR-derived probe. *

**Phenoloxidase** (PO)

Phenoloxidase (PO) participates in several insect physiological processes, including melanogenesis, cuticle sclerotization, wound healing, and other defense responses (2, 3). It catalyzes the formation of quinones that are precursors of melanin. Melanin and proteins may cross-link to form a capsule around invading parasites. Additionally, quinones might have antimicrobial effects (4). Proteolytic activation of phenoloxidase (proPO) in insects is mediated by a largely unknown serine proteinase pathway triggered by microbial surface molecules, such as lipopolysaccharide, peptidoglycan, and β-1,3-glucan (3, 5). ProPO-activating proteinase (PAP), also known as proPO-activating enzyme, cleaves proPO and causes its activation.

So far, we have isolated three PAPs from the tobacco hornworm, *Manduca sexta* (6–8). They all cleave proPO at Arg but require an auxiliary factor to generate active PO. We have purified and characterized the “cofactor” as a high *M* complex of serine proteinase homolog-1 and -2 (SPH-1 and -2) but do not yet understand its mechanism (9, 10). A similar phenomenon was reported in the beetle *Holotrichia diomphalia* (11). In contrast, *Bombyx mori* proPO-activating enzyme does not appear to need any cofactor for proPO activation (12).

Proteinase inhibitors in human plasma remove excessive proteinases (13). These include serine proteinase inhibitors of the serpin superfamily, whose typical sizes range from 370 to 450 residues (14, 15). To date, over 500 serpins have been identified in eukaryotes, bacteria, and viruses (16). They share a common tertiary structure albeit low sequence similarities of 25–50%. Many serpins have been purified from arthropods, including *B. mori*, *M. sexta*, *Aedes aegypti*, *Mythimna unipuncta*, and *Tachypeles tridentatus* (17–24). They regulate a number of biological processes including hemolymph coagulation, proPO activation, and induced synthesis of antimicrobial peptides. There are nearly 30 and 15 serpin genes in the *Drosophila melanogaster* and *Anopheles gambiae* genomes (25, 26), respectively.

In *M. sexta*, the serpin-1 gene encodes 12 variants through alternative exon usage (27–29). These serpins differ in their reactive center loops near the carboxyl terminus, which are encoded by variants of exons 9. The serpin-1J variant inhibits all three PAPs by forming SDS-stable complexes (8). *M. sexta* serpin-3 also blocks proPO activation by inhibiting PAP-1 and PAP-3 in the hemolymph (30). The detection of PAP-serpin-3 complexes in the induced hemolymph provided strong evidence supporting that serpin-3 is a physiological regulator of the two PAPs. *D. melanogaster* serpin-27A, orthologous to *M. sexta*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY572799.

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1 The abbreviations used are: PO, phenoloxidase; proPO, prophenoloxidase; PAP, proPO-activating proteinase; IEARpNA, acetyl-Ile-Glu-Ala-Arg-p-nitroanilide; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HP, hemolymph proteinase; SPH, serine proteinase homolog.
serpin-3, regulates melanization in vivo (31, 32). D. melanogaster Spn43Aac, which controls a putative serine proteinase caspase for the Toll pathway activation, also affects melanin deposition (33). Recently, we partially purified a new serpin (serpin-6) from M. sexta hemolymph (1). It regulated proPO activation by forming high Mr, SDS-stable complex with PAP-3 in vitro. However, M. sexta serpin-6 did not strongly inhibit PAP-1 or PAP-2.

Here, we report the cDNA cloning of M. sexta serpin-6 and its recombinant expression in Escherichia coli. Biochemical analysis demonstrated that recombinant serpin-6 inhibited PAP-3 efficiently. The mRNA and protein levels of serpin-6 were up-regulated in hemocytes and fat body after a bacterial challenge. Moreover, we characterized the hemolymph proteins bound to serpin-6.

**EXPERIMENTAL PROCEDURES**

Insects and Collection of Hemolymph, Hemocytes, and Fat Body—M. sexta eggs were purchased from Carolina Biological Supply, and the larvae were reared as described previously (34). Day 2 fifth instar larvae were injected with 1 × 10⁷ cfu/ml E. coli H316 and 10 mg/ml Nicotiana tabacum (Nicotiana alata) leaf extract and sacrificed at 48 h post-injection (7).

Cloning of Serpin-6 cDNA Fragments by PCR—To isolate M. sexta serpin-6 cDNA, degenerate primers (j801—j810) were designed based on its internal peptide sequences (1) for PCR amplification of the serpin cDNA fragments. Primers j801 (5’-GTC GGA GTT GTT RTT NGT CAT-3’) and j802 (5’-GTC GGA GTT RTT NGT CAT-3’) encode peptide 2 (MTNXNSDSYEFTTANK); primers j808 (5’-GTC ATG GAG GAA WSN TAY ATG-3’) and j809 (5’-GTC GGA ACW AAY ATH ATH-3’) encode peptide 1 (EVGDNIANINLTER); primers j803 (5’-GAC GCC TAY GAY TTY CAN AC-3’) and j804 (5’-GTC GGA GTT RTT NGT CAT-3’) encode peptide 2 (MTNNXNDSEYFTTANK); primers j805 (5’-GTC ATG GAG GAA WSN TAY ATG-3’) and j806 (5’-GAC GCC TAY GAY TTY CAN AC-3’) encode peptide 3 (LAAWMEMSYSM); primers j807 (5’-GAC GCC TAY GAY TTY CAN AC-3’) and j810 (5’-GTC GGA GAA GCC TTY TTG TTY CAT-3’) encode peptide 4 (TKKPFVSSRET).

PCR amplification was performed using primer pairs shown above in 25 µl of PCR reaction mixture containing 1 × 10⁻⁵ M dNTPs (2.5 mM each), 2.5 µM of MgCl₂, (25 mM), a pair of degenerate primers (25 pmol each), 10 ng of cDNA from the induced M. sexta larval fat body cDNA library (7), 0.5 µl of TaqDNA polymerase (5 units, Promega), and H₂O to bring the total volume up to 50 µl. Thermal cycling conditions were: 94 °C, 3 min and 35 cycles of 94 °C, 30 s; 50 °C, 40 s; and 72 °C, 60 s. After electrophoresis, PCR products were excised and recovered from the agarose gel using QIAQuick gel extraction kit (Qiagen). The fragments were individually ligated with pGem-T vector and transformed into high efficiency JM109 competent cells (Promega). Plasmid DNAs were isolated from the transformants, digested with NcoI-SphI and cloned into the same sites in plasmid H6pQE60 (35). To amplify a cDNA fragment encoding the mature serpin-6, a PCR reaction was performed using ClustalW 1.7 program from MacVector 6.5 software (Genetics Computer Group, 1998).

**Expression and Purification of Soluble Serpin-6 from E. coli Cells**—To amplify a cDNA fragment encoding the mature serpin-6, a PCR reaction was performed using forward primer (j815: 5’-TAA CCA TGG ATT GAT TTT CTC CC-3’) and reverse primer (j816: 5’-TAT GAC TGC TTA TTT CTT AGG GT-3’). The PCR product was digested with Ncol-SphI and cloned into the same sites in plasmid H6pQE60 (35). The resulting recombinant plasmid, serpin-6-H6pQE60, was sequenced to confirm correct insertion and sequence. The expression, extraction, and affinity purification of serpin-6 were performed under non-denaturing conditions as previously described (29, 35). The recombinant protein eluted from nickel-nitrilotriacetic acid-agarose (Qiagen) was adjusted with ammonium sulfate to a final concentration of 1.0 M. After centrifugation at 15,000 × g for 30 min, the cleared protein solution was separated by hydrophobic interaction chromatography on a 5-ml phenyl-Sepharose column (Amersham Biosciences) and eluted at 1.0 ml/min for 40 min with a linear, descending gradient of 1.0–0 M ammonium sulfate in 10 mM potassium phosphate, pH 6.4. Concentration and buffer exchange of the serpin-6 fractions were performed in a Centriprep-30 (Millipore). The purified protein was stored at −70 °C in 20 mM Tris-HCl, pH 7.5. A rabbit polyclonal antiserum was prepared against the recombinant serpin (Cocalico Biologicals Inc.).

**Determination of Cleavage Site in Serpin-6**—In vitro digestion of recombinant serpin-6 (12 µl) was carried out at room temperature for 10 min. The residual amidase activity was measured using 150 µl, 50 µM acetyl-Ile-Glu-Ala-Arg-p-nitroanilide (IAPRNAG) (7).

**Inhibition of ProPO Activation by Serpin-6**—In a Fraction of the Larval Hemolymph—The induced hemolymph was fractionated with 0–50% saturation of ammonium sulfate to obtain the proPO activation system (29). Recombinant serpin-6 (10 µl) at final concentrations of 0–150 µg/ml was incubated with the plasma fraction (10 µl, 1:5 diluted in H₂O) in the presence of M. sexta (1 µl, 1 µg/ml). As controls, 10 µl of buffer for the fraction without M. sexta were added individually and incubated at room temperature for 10 min prior to the PO activity assay.

**Kinetic Inhibition Assay**—A progressive curve method was employed to determine the association rate constant (37). Purified PAP-3 (1.8 µM) was mixed with IAPRNAG solutions containing serpin-6 at different molar ratios. Absorbance at 405 nm was monitored in the kinetic mode with a Bio-Rad laboratories VERSAmax microplate reader (Molecular Devices). The progressive curves were first analyzed according to Equation 1: $v = k₀(1 - e^{-kt})$, where $k₀$ is the pseudo-first-order rate constant of inhibition, and $V₀$ is the initial velocity. The experimental values were fit into the equation using Prism 3.0 (GraphPad Software, Inc.). From the plot of $v/k₀$ versus $[I]$, $kₚ$ was derived from a linear regression analysis of the data according to Equation 2: $kₚ = k₀/β[1 + [S]/Kₘ]$. For calculating the inhibition constant ($Iₚ$), the values of PAP-3, $t_{½}$, and $V₀$ were derived from SDS-PAGE, immunoblot analysis, and densitometry using the purified recombinant serpin-6 as a standard (35).

**Detection of Serpin-Enzyme Complexes by Immunoblot Analysis**—PAP-3 (1 µl, 100 µg/ml) and recombinant serpin-6 (2 µl, 100 µg/ml) were mixed with 100 µg/ml rabbit anti-PAP-3 polyclonal antiserum (in naive and induced larval hemolymph, the plasma concentrations of serpin-6 were estimated by SDS-PAGE, immunoblot analysis, and densitometry using the purified recombinant serpin-6 as a standard (35).

**RNA Extraction and Reverse Transcription-PCR Analysis**—Total RNA samples were extracted from fat body and hemocytes of naive and induced M. sexta larvae using Micro-to-Midi total RNA purification system (Invitrogen). First-strand cDNA synthesis was performed using 2–4 µg total RNA, 10 µmol oligo(dT)₁₇, and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) at 37 °C for 1 h. M. sexta ribosomal protein S3 cDNA was used as an internal standard to normalize the templates in a preliminary PCR experiment. After template adjustment, PCRs were performed to detect relative levels of serpin-6 cDNA. The primers used were j812 (5’-TGA TGA CGT CTG TCT TCT CTC CTG CT-3’) and j813 (5’-TGC TCT CTC CTA-3’) for serpin-6 and k504 (5’-CGG GTT AGG ACT CTG GTC-3’) and k501 (5’-AAC TCA TGA CGC TGC-3’) for serpin-3 and k505 (5’-GCC GTT CCT GCC CTG TT-3’) for ribosomal protein S3. The thermal cycling conditions were: 94 °C, 30 s; 50 °C, 30 s; 72 °C, 60 s. PCR cycle numbers were empirically chosen to show comparable band intensity and avoid saturation. After separation by 1.5% agarose gel electrophoresis, intensities of the PCR products were quantified using Kodak Digital Science one-dimensional gel analysis software (35).
plasma at final concentrations of 1/27,000, diethylthiocarbonate, and 1-phenyl-2-thiourea were added to the samples, which were incubated at room temperature for 30 min, to activate the serine proteinase system yet prevent hemolymph proteinases,2 added to terminate the coupling reaction for another 2 h. For isolating serpin-6-proteinase complexes, 15 ml of cell-free hemolymph was collected from naive or bacteria-injected larvae (fifth instar, day 3).

The monoisotopic peptide masses were also compared with the theoretical masses of serpin-6 (Fig. 1). The open reading frame in the 3'-untranslated region (Fig. 1). A polyadenylation signal in italics, above the nucleotide sequence, indicates the termination of short form of serpin-6. Substitutions of nucleotides in the open reading frame are underlined. A non synonymous substitution (A→C) changes Thr\textsuperscript{109} to Asn\textsuperscript{109}.

Immunoadfinity Chromatography and MALDI-TOF Mass Finger-print Analysis—To couple serpin-6 antibodies to protein A-Sepharose, 4.8 ml of the rabbit antiserum, 2.4 ml of resin (Sigma), and 1.0 M sodium phosphate, pH 8.0. The resin was washed with 10 volumes of 0.2 M, and 1 mM, respectively, to activate the serine proteinase system yet prevent hemolymph proteinases,2 added to terminate the coupling reaction for another 2 h. For isolating serpin-6-proteinase complexes, 15 ml of cell-free hemolymph was collected from naive or bacteria-injected larvae (fifth instar, day 3).

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RESULTS

Molecular Cloning and Structural Features of M. sexta Serpin-6—We designed degenerate primers encoding five internal peptides of M. sexta serpin-6 (1). Because the order of these peptides was unknown, we used different combinations of the primers to amplify cDNA fragments by PCR and cloned the products. Sequence analysis confirmed the cDNA inserts encoded different regions of a serpin. Using the longest one (642 bp, amplified by j802 and j803) as a probe, we screened 105 plaques in an induced fat body cDNA library and identified 78 positives. We carried out plaque purification and in vivo excision for five of the positive clones. Complete sequence analysis indicated that their cDNA inserts were 1.5 or 2.1 kb. The long form contained an additional 0.6-kb sequence spanning nucleotides 113–1531. Its corresponding protein is 412 residues long and includes the five peptide sequences of the mature protein (395 residues) has a calculated molecular mass of 44,957 Da, smaller than the experimental value (46,710 ± 10 Da). The difference (−1153 Da) may result from N-linked glyco
The calculated isoelectric point of serpin-6 is 6.1, higher than the experimental value of 5.4.

A BLAST search of GenBank™ revealed that *M. sexta* serpin-6 is most similar in amino acid sequence to *D. melanogaster* serpin-5 and *A. gambiae* serpin-9 (Fig. 2). It is 39% identical and 55% similar to *Drosophila* serpin-5, which may regulate the Toll and Imd pathways (38, 39).

*M. sexta* serpin-6 is 36% identical and 53% similar to *A. gambiae* serpin-9 but only 25 and 27% identical to *M. sexta* serpin-1J and serpin-3, respectively. While the reactive center loops of serpins are hypervariable, our multiple sequence alignment showed that the loop sequences of *M. sexta* serpin-6, *D. melanogaster* serpin-5, and *A. gambiae* serpin-9 are strikingly similar (Fig. 2). The predicted scissile bond is located between Arg and Ser residues, suggesting that these three serpins inhibit proteinases with a trypsin-like specificity (e.g. PAP-3). The loop sequences are less similar to the corresponding regions in *M. sexta* serpin-1J and serpin-3.

*M. sexta* serpin-3 contains a 20-residue extension at the amino terminus, which is absent in the other serpins.

Increases in Serpin-6 mRNA and Protein Levels after a Bacterial Challenge—To understand the transcriptional regulation of serpin-6 gene in response to a microbial infection, we analyzed the relative mRNA levels in the normalized total RNA samples by reverse transcription-PCR (Fig. 3A). While the faint bands in naïve hemocytes and fat body represented a low, constitutive level of serpin-6 transcripts, a significant up-regulation was observed in both tissues after a bacterial challenge. Additionally, we examined the serpin-6 concentration in hemolymph by immunoblot analysis (Fig. 3B). A low level of serpin-6 was present in naïve *M. sexta* larval hemolymph. After injecting the larvae with killed *E. coli* or *M. luteus*, we detected a small decrease of the 46-kDa protein after 6 h, and the band intensity was higher than the control at 24 h post-injection. The observed protein level changes probably reflected consumption and replenishing of serpin-6 after the bacterial challenge. The concentrations of serpin-6 in naïve and induced hemolymph are estimated to be 15 and 30 μg/ml, respectively.

Purification and Characterization of Recombinant Serpin-6—To explore its biochemical functions, we produced serpin-6 in an *E. coli* expression system. The soluble serpin-6 (15 μg/ml, ~50% of the total serpin-6 produced) was purified to near homogeneity by nickel affinity and hydrophobic interaction chromatography. Serpin-6 migrated as a 44-kDa single band on a 10% SDS-PAGE under the reducing condition (Fig. 4). Trypsinolytic peptide mass fingerprint analysis of the recombinant protein revealed 39 matching peptides, representing 77% of the overall sequence including the carboxyl-terminal fragment (data not shown).

A characteristic feature of a serpin-proteinase reaction is consumption and replenishing of serpin-6 after the bacterial challenge. The concentrations of serpin-6 in naïve and induced hemolymph are estimated to be 15 and 30 μg/ml, respectively.

**FIG. 2.** Sequence alignment of five insect serpins. The amino acid sequences of *M. sexta* serpin-6, serpin-1J, serpin-3, *D. melanogaster* serpin-5, and *A. gambiae* serpin-9 are aligned. Amino-terminal secretion sequences are underlined. The predicted P1 residue before the scissile bond is double underlined. The reactive site loop extends from P16 to P5. Asterisk, identical; dot, conservative substituted.

**FIG. 3.** Changes in PAP-3 mRNA and protein levels in bacteria-challenged *M. sexta* larvae. A, total RNA samples from hemocytes (CH, IH) or fat body (CF, IF) of control or induced insects (24 h after injection with H₂O or *E. coli*, respectively) were analyzed by reverse transcription-PCR as described under “Experimental Procedures.” The *M. sexta* ribosomal S3 mRNA was used as an internal standard to demonstrate equal amount of RNA templates. B, cell-free hemolymph samples (2 μl) collected at 1, 6, 12, and 24 h after fifth instar day 2 larvae had been injected with 1 × 10⁷ killed *E. coli* cells (lanes 2–5) or 100 μg *M. luteus* (lanes 6–9) were subjected to 10% SDS-PAGE analysis under the reducing condition. Lane 1 was the sample at 0 h. Immunoblot analysis was performed using 1:2000 diluted serpin-6 antiserum as the first antibody.
formation of a high $M_r$, SDS-stable complex of the serpin and its cognate enzyme. We conducted an experiment to test whether serpin-6 can form such a complex with PAP-3. In the control of serpin-6 or PAP-3 only, PAP-3 antibodies recognized the two polypeptide chains of the proteinase but not serpin-6 (Fig. 5A). After the two proteins were incubated together, the light chain of PAP-3 remained at the 21-kDa position, but the 37-kDa catalytic domain completely disappeared. Instead, a new immunoreactive band migrated to the 75-kDa position anticipated for a complex of serpin-6 and PAP-3 catalytic domain. This 75-kDa band was also recognized by serpin-6 antibodies (Fig. 5B). Therefore, the recombinant serpin-6 was an active inhibitor that formed a covalent complex with PAP-3.

After PAP-3 was incubated with the recombinant serpin at different molar ratios, the residual amidase activity decreased linearly as serpin-6 concentration increased (Fig. 6, A and B). Complete inhibition occurred at a molar ratio of 2:1 (serpin-6: PAP-3). Moreover, recombinant serpin-6 also blocked proPO activation by PAP-3 and SPHs. To test whether serpin-6 could inhibit proPO activation in hemolymph, we obtained the 0–50% ammonium sulfate fraction of $M. sexta$ plasma, which contained all the components for proPO activation. Added serpin-6 at 45 and 140 ng/μl blocked proPO activation by 50 and 90%, respectively (Fig. 6C). This result suggested that serpin-6 inhibits one or more of the serine proteinases in the proPO activation system.

To further characterize the serpin-proteinase reaction, we
determined the second-order association rate constant ($k_a$) for the inhibition (Fig. 7). The $k_a$ ($2.6 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$) indicated that serpin-6 may contribute to the inhibitory regulation of PAP-3 in hemolymph.

We determined the cleavage site of serpin-6 upon reacting with PAP-3 by MALDI-TOF mass spectrometry (Fig. 8). A major peak of 4639 Da was detected in the proteinase-inhibitor mixture but not in the control spectra of serpin-6 and PAP-3 only. This peak had exactly the same mass as the carboxy-terminal peptide released from a cleavage of serpin-6 between Arg373 and Ser374.

**Isolation and Characterization of Hemolymph Proteins Associated with Serpin-6**—We activated the induced larval hemolymph with *M. luteus* and examined whether or not serpin-6 and PAP-3 form a complex in plasma. Immunoaffinity chromatography using serpin-6 antibodies allowed us to isolate serpin-6 and its associating proteins from the plasma sample. We analyzed the proteins eluted from the affinity column by immunoblot (Fig. 9) and trypticolytic peptide mass fingerprint analyses. While most serpin-6 remained intact, part of it was either cleaved or covalently linked with PAP-3 or hemolymph proteinase 8 (HP8) (Fig. 9, B–D). Formation of the serpin-proteinase complexes markedly increased in the induced hemolymph after a bacterial elicitation. The peptide mass fingerprint of the upper band had 12 mass fits with serpin-6 and 8 with the PAP-3 catalytic domain (Fig. 9A). The lower band represents the complex of serpin-6 and HP8: 11 and 5 mass peaks match those calculated from serpin-6 and HP8 proteinase domain sequences, respectively. These results indicate that serpin-6 is a physiological regulator of PAP-3 and HP8 during immune responses.

Immunoblot analysis indicated that several other immune proteins also tightly associated with the antibody column. These include: *M. sexta* immulectin-2, proPO, PO, SPH-1, SPH-2, and HP14 (Fig. 9, E–I). Peptide mass fingerprint analysis confirmed the presence of these proteins. For instance, peptide map of the 37-kDa immunoreactive band (Fig. 9E) covers 40% of immulectin-2 sequence. Additionally, 13 trypsinolytic peptides of the 15-kDa band (Fig. 9A) match 58% of attacin-2 sequence. While similar amounts of serpin-6 and immunlectin-2 were present in the naive and induced hemolymph, we isolated more attacin-2, proPO, PO, SPHs, HP14, and serpin-6-proteinase complexes from the induced hemolymph (Fig. 9A). This result suggests that the secondary defense reactions involve more molecules in hemolymph from the bacteria-challenged larvae.

**DISCUSSION**

It is common in vertebrates and invertebrates that extracellular serine proteinase cascades mediate acute-phase responses upon microbial infection. These pathways are often regulated by irreversible inhibitors of the serpin superfamily (15, 40, 41). In this study, we have cloned and characterized the newly discovered serpin-6 from *M. sexta* hemolymph. It is constitutively synthesized in the larval fat body and hemocytes. After a microbial challenge, induced transcription and translation of serpin-6 gene lead to a higher protein level in the plasma. Recombinant serpin-6 formed covalent complex with PAP-3 and inhibited proPO activation by the purified enzyme and in the hemolymph. The *in vivo* half-life of PAP-3, calculated as $1/k_a$, is 2.4 min in naive larval hemolymph and 1.2 min in induced hemolymph. Endogenous serpin-6 was identified in an SDS-stable complex with PAP-3 in the hemolymph after *M. luteus* stimulation. These results provide good evidence that serpin-6 contributes to the regulation of PAP-3 under physiological conditions. Along with our previous work on *M. sexta* serpin-1J and serpin-3 (8, 29, 30), we demonstrate in this report that multiple serpins inhibit a single proteinase in the insect hemolymph. Comparative kinetic analysis should allow us to evaluate relative importance of these three serpins in the regulation of PAP-1, PAP-2, and PAP-3.

Considering the low sequence conservation of the serpin family, we believe the high similarity among *M. sexta* serpin-6, *D. melanogaster* serpin-5, and *A. gambiae* serpin-9 is significant. In particular, their P4–P4′ residues are nearly identical in this typically hypervariable region (Fig. 2). Since this is the site where serpins specifically interact with their target enzymes, we anticipate that *M. sexta* serpin-6 and the dipteran serpins may perform similar physiological functions. The expression profile of serpin-6 appears to be consistent with that of *Drosophila* serpin-5, a potential regulator of the Toll and Imd pathways (39, 42).

In contrast, serpin-6 is only 25 and 27% identical in sequence to serpin-1J and serpin-3, respectively. Its reactive center loop is 2 residues shorter. The P4–P4′ region of serpin-6 (Phe-Gly-Phe-Arg-Ser-Arg-Pro) is remarkably different those of serpin-1J (Leu-Thr-Asp-Arg-Cys-Cys-Ser-Asp) and serpin-3 (Ile-Gln-Asn-Lys-Phe-Gly-Glu-Asp), except for the positively charged residue at the P1 site, which fits the negatively charged primary specificity pocket of PAPs. The P4–P4′ regions in *M. sexta* serpin-3 and its orthologs from *H. cunea* and *M. domestica* best resemble the proPO sequences around the cleavage activation site (31, 32, 43).

Since serine proteinase genes greatly outnumber serpin genes in the *D. melanogaster* and *A. gambiae* genomes, we postulated that a single insect serpin may regulate multiple serine proteinases in one or more pathways (1). Genetic analyses provide support for such hypothesis: loss-of-function mutations in a serpin gene can lead to pleiotropic phenotypes (31–33). In this paper, we present direct biochemical evidence that serpin-6 regulates two proteinases (i.e. PAP-3 and HP8) in the hemolymph. While the involvement of PAP-3 in proPO activation is established, we have not yet elucidated the biological function of HP8. Of the 18 hemolymph proteinases we recently cloned, HP8 is most similar in sequence to *H. di-
omphalia proPO-activating factor-III, a Ca$^{2+}$-dependent activating enzyme for proPO-activating factor-II precursor (44). Further analysis is required to test the effect of serpin-6 on blocking the proteolytic processing of *M. sexta* proSPH-1 and proSPH-2, as a step toward understanding the physiological roles of serpin-6 in regulating proPO activation. A high Mr complex of SPH-1 and SPH-2 is structurally similar and functionally equivalent to *H. diomphalia* proPO-activating factor-II.

Association of hemolymph proteins to the serpin antibody column provides us a unique opportunity to examine a macromolecular complex formed in the insect defense response. This complex associated with serpin-6 specifically, since we did not detect any of these proteins bound to the control column coupled with the preimmune antibodies. Due to the stringent washing conditions and absence of abundant hemolymph proteins in the eluate, direct or indirect associations of these proteins with serpin-6 appear to be strong and specific. Additionally, the composition of such complex appears to be quite stable. Many of these proteins were also identified in the bound fractions from the immunoaffinity columns for *M. sexta* serpin-4 and serpin-5, negative regulators of the proPO activation system (46).

Among the co-purified proteins, immulectin-2 bound to lipopolysaccharide of Gram-negative bacteria and stimulated the proPO activation (45). SPH-1 and SPH-2, which associated with immulectin-2 (9), were also identified as major proteins in this experiment (Fig. 9, E, G, and H). These two proteins are required by PAP to generate active PO. ProPO and PO were detected in the complex and so was HP14, an initiating protease of the proPO activation system (Fig. 9, F and I). While these proteins all participate in melanization, we have also co-eluted several minor components that may be also involved in proPO activation and/or other defense reactions. These include HP17 and HP21.3

Much to our surprise, *M. sexta* attacin-2 in the induced hemolymph also associated with the antibody column (Fig. 9A); we expected antimicrobial peptides to directly and independently interact with bacterial surfaces. The detection of attacin-2 suggests that recognition specificity and binding strength of the immune protein complex may be enhanced by associating with a bacteria-killing protein.

Taken together, our results strongly suggest that a bacterial entry may elicit a subset of hemolymph proteins to associate and form a non-covalent protein complex. The molecular interactions among pattern recognition proteins, HPs, SPHs, proPO, and other proteins in the complex ensure a localized defense reaction against the invading organisms. After exerting their functions, active serine proteinases are inhibited by multiple serpins to limit potential damage to the host tissues and cells. Serpin affinity chromatography has allowed us to observe the immune complex for the first time. Further analyses of this complex may lead to the elucidation of proPO activation cascade and other immune proteinase pathway in *M. sexta*.

3 Z. Zou and H. Jiang, unpublished data.
4 Y. Tong, H. Jiang, and M. Kanost, unpublished results.
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