Manduca sexta serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases

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Summary

Many serine proteinase inhibitors of the serpin superfamily have evolved in vertebrates and invertebrates to regulate serine proteinase cascades that mediate the host defense responses. We have isolated an immune-responsive serpin from the tobacco hornworm, Manduca sexta. This inhibitor, M. sexta serpin-3, contains a reactive site loop strikingly similar to the proteolytic activation site in prophenoloxidase (proPO). Molecular cloning and sequence comparison indicate that serpin-3 is orthologous to Drosophila melanogaster serpin 27A, a regulator of melanization. M. sexta serpin-3 is constitutively present in hemolymph at a low concentration of 5-12 µg/ml and increases to 30-75 µg/ml after a microbial challenge. Recombinant serpin-3 efficiently blocks proPO activation in the hemolymph, and it forms SDS-stable acyl-enzyme complexes with purified proPO-activating proteinases (PAPs) from M. sexta. PAP-serpin-3 complexes were isolated by immunoaffinity chromatography from hemolymph activated by treatment with Micrococcus luteus. Kinetic analysis of PAP-serpin-3 association strongly suggests that serpin-3 is a physiological regulator of the proPO activation reaction.
INTRODUCTION

Synthesis of melanin by insects and other arthropods in response to infection results from a proteolytic cascade triggered by microbial cell wall components (1-3). In this innate immune response, microbes are recognized as foreign when soluble pattern recognition proteins bind to bacterial peptidoglycan or lipopolysaccharide or fungal β-1,3 glucan, initiating activation of a series of proteinases. Phenoloxidase (PO), the final enzyme in this cascade, exists in hemolymph as an inactive zymogen, prophenoloxidase (proPO), which is activated by a specific proteinase. Active PO catalyzes the hydroxylation of monophenols to o-diphenols and oxidation of o-diphenols to quinones that can polymerize to form melanin. Cytotoxic molecules produced in this process, including quinones and reactive oxygen intermediates, may kill the invading microorganisms that are trapped by melanin (4).

Our understanding of the proPO activation cascade is still incomplete, and the number of proteolytic steps in the pathway is unknown. However, serine proteinases that directly activate proPO, known as proPO activating enzyme (PPAE) or proPO-activating proteinase (PAP), have been isolated and cloned from several arthropod species, including the silkworm, Bombyx mori (5, 6), the tobacco hornworm, Manduca sexta (7-9), a beetle, Holotrichia diomphalia (10, 11), and a crayfish, Pacifastacus leniusculus (12, 13). These enzymes are composed of a carboxy-terminal serine proteinase domain and at least one clip domain at their amino-terminus. A specific proteolytic cleavage between the domains activates the proteinase zymogens, but the two resulting chains remain covalently linked by a disulfide bond (14). The activated proteinase cleaves proPO at a conserved Arg-Phe bond at approximately residue 50 and leads to its activation (6-13).

Three PAPs from M. sexta have been identified and cloned as cDNAs. PAP-1 contains
one clip domain (7), whereas PAP-2 and PAP-3 each have two clip domains (8, 9). PAP-1 and PAP-3 are expressed as zymogens at a low constitutive level in naive larvae. Bacterial challenge induces synthesis of all three of the proPAPs, whose concentrations increase dramatically in the hemolymph (7-9). PAP-2 and PAP-3 also accumulate in the hemolymph of prepupae, and PAP-1 is present in the cuticle at the same stage.

The PAPs are synthesized as inactive zymogens and require activation by as yet unidentified proteinases upstream in the proPO cascade pathway. Once the PAPs are active, it is likely that they are regulated by specific inhibitors, because the quinones and reactive oxygen species generated by uncontrolled PO activation would be harmful to the host insect (1, 4). Potential candidates for regulators of PAPs are members of the serpin superfamily, which include proteins known to regulate serine proteinase cascades involved in mammalian inflammatory responses, coagulation, complement activation, and fibrinolysis (15, 16). Serpins, present in hemolymph of insects, including M. sexta (17), are proteins of approximately 45 kDa with a solvent-exposed reactive site loop (RSL) near their carboxyl-terminus. In an inhibition reaction, hydrolysis of the P1-P1’ peptide bond is accompanied by formation of a stable acyl intermediate, with the P1 residue of the serpin linked to the catalytic Ser residue of the proteinase. The cleaved serpin undergoes a large conformational rearrangement, in which the RSL inserts into a $\beta$-sheet in the serpin (16).

In M. sexta, two serpin genes have been identified (18, 19). The serpin-1 gene contains ten exons, with 12 alternative forms of exon 9. Alternative mRNA splicing generates twelve serpin variants identical in their amino-terminal 336 residues, encoded by the first eight exons, and a variable carboxyl terminus encoded by one of the exon 9 forms (20). Because exon 9
encodes the reactive center region, serpin-1 variants with different reactive site sequences have different inhibitory selectivity (21). The serpin-1 gene is constitutively expressed in fat body at a high level and, to lesser degree, in the granular hemocytes (22). The serpin-1 proteins are secreted into the plasma. *M. sexta* serpin-2 is expressed in cytoplasm of granular hemocytes in response to bacterial infection, but its function is not yet understood (19). There is also evidence for the existence of additional serpins in *M. sexta*, which are possibly encoded by different genes and may regulate the cuticular proPO activation (23).

Here we report the molecular cloning and analysis of an immune-responsive serpin (serpin-3) from *M. sexta*. Serpin-3, whose expression is stimulated by microbial infection, was shown to block the PPO activation cascade and to inhibit *M. sexta* prophenoloxidase activating proteinases.

**EXPERIMENTAL PROCEDURES**

*Insects* – *M. sexta* eggs were originally obtained from Carolina Biological Supply. Larvae were reared as described by Dunn and Drake (24).

*Collection of hemolymph, hemocytes and fat body from M. sexta larvae* – For determining the induction pattern of serpin-3 protein in plasma, day 2 fifth instar larvae were injected with 10 μl of filter-sterilized saline (0.85% NaCl), 10 μg/μl *Micrococcus luteus* (Sigma), 2×10⁶ cells/μl *Escherichia coli*, or 5×10⁵ cells/μl *Saccharomyces cerevisiae*. Hemolymph was collected into microcentrifuge tubes at 0, 1, 2, 6, 12, 24, 32, and 48 h after injection by cutting the dorsal horn. A few crystals of 1-phenyl-2-thiourea were added to each tube to prevent hemolymph melanization. Hemocytes were removed by centrifugation at 12,000×g for 10 min at 4°C. For total RNA preparation, hemocytes and fat body were collected
from larvae at 24 h after injection as described previously (25).

**Molecular cloning and sequence analysis** – A serpin-3 cDNA fragment, recovered from clone M5 (accession # BI262710) (26), was labeled with [α-32P] dCTP and used as a probe to screen a bacteria-induced *M. sexta* fat body cDNA library in Uni-Zap XR (Stratagene) (27). The positive plaques were purified and subcloned by in vivo excision. The nucleotide sequence of the longest cDNA insert was determined from both strands using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequences were edited with IBI PUSTELL programs. The amino acid sequence deduced from the cDNA was used to search the non-redundant protein database with the BLAST program (28). The most similar sequences were retrieved and aligned using the PILEUP program from the GCG Sequence Analysis Software Package (29). The signal peptide was predicted using the program of Nielsen et al. (30).

**Detection of an intron in the serpin-3 gene** – *M. sexta* genomic DNA (0.2 µg) was amplified by PCR in 30 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min. The forward primer (5'-TCA AAG GTG TTC CAG AGA AC-3') and reverse primer (5'-ATT GTT TTG CGT GTG GCT TTC-3') were located in the serpin-3 cDNA corresponding to 3' end of exon 8 and the 5' end of exon 10 in the serpin-1 gene, respectively. The PCR product was cloned and sequenced to reveal the sequence of the corresponding region of the serpin-3 gene.

**Expression of recombinant serpin-3 in E. coli** – The serpin-3 cDNA encoding the mature protein from residue 1 to 435 was amplified by PCR using a forward primer (5'-TCT CCC ATG GAC GAC GTC GAC-3') that contains an NcoI site and a reverse primer (5'-TGC GCT GCA GCT CGT TTC-3') that contains a PstI site. The PCR product digested with NcoI and PstI was purified by agarose gel electrophoresis and cloned into the same sites in H6pQE60 (31).
Serpin-3 was expressed in a one l culture of *E. coli* strain XL1blue carrying the recombinant plasmid. Serpin-3 was expressed in an insoluble form and therefore was purified under denaturing conditions by nickel-affinity chromatography (32). An aliquot of the purified recombinant serpin-3 (1.5 mg) was further resolved by preparative SDS-PAGE and used to produce a polyclonal rabbit antiserum.

*Production of soluble serpin-3 in a baculovirus expression system* – The *Bam* *HI*-Xhol cDNA fragment, containing the complete open reading frame for serpin-3, was inserted into pFastBac1 (Invitrogen Life Technologies). A recombinant baculovirus was generated from the resulting plasmid and amplified according to the manufacturer’s instructions. For producing soluble serpin-3, *Spodoptera frugiperda* Sf9 cells (2 × 10⁶ cells/ml) in 250 ml Sf-900 II serum-free medium were infected with the virus at a multiplicity of infection of 4 and incubated at 27°C with shaking at 140 rpm for 72 h. The conditioned medium was clarified by centrifugation at 500×g for 10 min, and the supernatant was placed in dialysis tubing and concentrated to 28 ml by covering the tubing with polyethylene glycol 8000, then dialyzed twice against 1.0 liter buffer A (20 mM Tris-Cl, 10 mM NaCl, pH 7.5) overnight at 4°C. The protein solution was applied to a 12-ml Q-Sepharose column and washed with 60 ml buffer A. The bound proteins were eluted with linear gradients of 10-220 mM NaCl (150 ml) and then 220-500 mM NaCl (50 ml) in buffer A. Fractions (2 ml each) were analyzed by 10% SDS-PAGE (33) and immunoblotting using serpin-3 antiserum as the first antibody. Concentration of the purified protein was determined by SDS-PAGE analysis along with a series of diluted ovalbumin standards. Intensity of the protein bands was digitized using Kodak Digital Science 1D gel analysis software. The amino terminal sequence of serpin-3 was determined by automated Edman degradation as described before (7).
**Northern blot analysis and RT-PCR** – Total RNA samples were isolated from hemocytes or fat body using a Rapid Total RNA Isolation Kit (Eppendorf-5 Prime, Inc). Northern blot analysis was performed using 20 µg RNA for each sample (8). The expression profile of serpin-3 was examined by RT-PCR using SUPERSCRIPT One-Step RT-PCR kit and PLATINUM Taq DNA polymerase (Invitrogen Life Technologies). At 0.5, 1, 2, 4, 6, 8, 12, 24 h after injection with 100 µg *M. luteus*, fat body from four larvae in each group was dissected and pooled for total RNA isolation. One µg of each RNA sample was treated with 2 units of DNase I to remove trace amounts of genomic DNA. Reverse transcription was performed at 45°C for 30 min, followed by PCR amplification with 20 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s using gene-specific primers for serpin-3 (5′-GAT TCC TCG CGA TTC GAT GC-3′ and 5′-CAT TTA CGT CAT TAA GTT TCA TG-3′). Primers for ribosomal protein S3 (5′-CGC GAG TTG ACT TCG GT-3′ and 5′-GCC GTT CTT GCC CTG TT-3′) were used in the RT-PCR as an internal control. PCR products were analyzed by agarose gel electrophoresis with detection by ethidium bromide staining.

**Proteinase inhibition assays** – *M. sexta* proPAP-1 and proPAP-3 produced as recombinant proteins by baculovirus expression (34) contained spontaneously activated PAP-1 and PAP-3, which were separated from the zymogen forms by anion exchange chromatography (data not shown). Concentrations of the active PAPs were determined as described above for serpin-3. The purified PAP-1 or PAP-3, each at 50 nM, was preincubated with serpin-3 at different molar ratios (0.4:1 to 4:1) in 10 µl buffer B (0.1 M Tris-HCl, pH 8.0, 0.03% BSA) for 10 min at room temperature. Residual amidase activity was assayed by adding 190 µl of 50 µM N-acetyl-Ile-Glu-Ala-Arg-p-nitroanilide (IEAR-pNA) in buffer C (50 mM Tris-HCl, pH 7.8, 50 mM NaCl)
Determination of the association rate constants ($k_{assoc}$) – The $k_{assoc}$ for PAP-1 and serpin-3 was determined under second order conditions according to Beatty et al. (35). Briefly, recombinant PAP-1 and serpin-3, each at 16 nM, were incubated in 18 µl buffer B at room temperature. At each designated time ($t$) (15-90 sec), 182 µl of 50 µM IEARpNa in buffer C was added to determine the residual amidase activity ($v$). Half-life of the inhibition reaction ($t_{0.5}$) was determined by plotting $1/v$ against $t$, and the $k_{assoc}$ was calculated according to $k_{assoc} = 1/t_{0.5} × [E_0]$ (35). The $k_{assoc}$ for PAP-3 and serpin-3 was also determined under second order conditions with 8.8 nM PAP-3 and 38.6 nM serpin-3. The $k_{assoc}$ was calculated using the standard equation: $k_{assoc}t = [\ln (1 + (I_0 - E_0)/E_t) - \ln (I_0/E_0)]/(I_0-E_0)$ (36).

Regulation of proPO activation in hemolymph by serpin-3 – A preparation of hemolymph proteins that contains the components of the proPO activation cascade (proPO activation fraction) was produced as described previously (21). Ten µg of M. luteus in 1 µl sterile saline was added to 100 µl of PPO activating fraction (diluted 1:5 in water). At different incubation times (0-60 min), 10 µl aliquots of the reaction mixture were added to 700 µl phenoloxidase substrate solution (2 mM 3-hydroxytyramine in 50 mM sodium phosphate, pH 6.8) to measure PO activity (37). To test the inhibition of proPO activation, serpin-3 at final concentrations of 0-61 µg/ml was added to 1µ5 diluted proPO activation fraction (10 µl), along with 1 µg M. luteus. PO activity was determined after incubation for 10 min at room temperature.

For detecting the formation of serpin-PAP complex, purified serpin-3 was reacted with the purified PAP-1 or PAP-3 in buffer B at a molar ratio of 1:1 or 1:2 (serpin/proteinase).
control samples, diisopropyl fluorophosphate (DFP) at a final concentration of 5 mM was mixed with PAPs prior to the addition of serpin-3. After incubation at room temperature for 10 min, the reaction mixtures were treated with SDS sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. Proteins transferred onto a nitrocellulose membrane were subjected to immunoblot analysis using 1:3000 diluted antisera against PAP-1 (7), PAP-3 (9), or serpin-3 as the first antibody.

Detection of serpin-PAP complexes in hemolymph by affinity chromatography – A serpin-3 immunoaffinity column was prepared according to Harlow and Lane (38). Briefly, 2.4 ml protein A-Sepharose beads (Sigma) were incubated with 4.8 ml serpin-3 antiserum in 19.2 ml phosphate-buffered saline (PBS; 0.8% NaCl, 0.02% KCl, 0.144% NaH$_2$PO$_4$, 0.024% KH$_2$PO$_4$, pH 7.4) for 1 h at room temperature to allow binding of the antibodies to protein A. Following a washing step, covalent coupling of antibodies to protein A was carried out by adding 20 mM dimethylpimelimidate in 0.2 M sodium borate, pH 9.0. After incubation at room temperature for 2 h, the reaction was stopped by incubating the beads with 0.2 M ethanolamine at pH 8.0.

Hemolymph from day 2 fifth instar larvae was collected and centrifuged to remove hemocytes. The plasma was divided into two aliquots (2 ml each). DFP was immediately added to one aliquot at a final concentration of 5 mM. *M. luteus* (100 µg) was added to the other aliquot, which was incubated for 5 min before addition of DFP. The two samples were then separately incubated with 0.6 ml of affinity beads in 3.4 ml PBS overnight at 4°C. The resin was packed into two columns and washed with 20 ml of 10 mM sodium phosphate, pH 8.0. The bound proteins were eluted by sequential applications of 0.3 ml of 100 mM glycine-HCl, pH 2.5 with collection of the eluents into 60 µl, 1 M sodium phosphate, pH 8.0. The fractions were
subjected to immunoblot analysis as described above.

RESULTS

cDNA cloning and sequence of serpin-3— From a subtractive *M. sexta* cDNA library representing genes expressed in larval fat body in response to bacterial challenge (24), we isolated a cDNA clone (M5, Genbank accession number BI262710) that encoded a partial amino acid sequence highly similar to members of the serpin superfamily. Using the 527 bp cDNA fragment as a probe, we screened a bacteria-induced *M. sexta* fat body cDNA library, isolated 35 positives clones from $8 \times 10^4$ plaques screened, and sequenced the two longest cDNA inserts (Fig. 1). The nucleotide sequence of serpin-3A and serpin-3B cDNAs differed slightly, with several substitutions, deletions and insertions, mostly occurring in the 3' untranslated region. The serpin-3B cDNA (2498 bp) is 13 bp longer in the 3' untranslated region than serpin-3A (2485 bp) (data not shown). A polyadenylation signal, AATAAA, was present 24 bases upstream of a poly(A) tail in both cDNAs. The open reading frames of the two serpin-3 cDNAs were identical in both length and their deduced amino acid sequences, since the eight substitutions in the open reading frame were silent. The encoded polypeptide is 435 residues long with a predicted signal peptide comprising the first 20 residues. Four potential N-glycosylation sites are present at Asn$^{38}$, Asn$^{188}$, Asn$^{227}$, and Asn$^{294}$. The calculated molecular mass of the mature protein, beginning at residue 21, is 48,937 Da, and the calculated isoelectric point is 5.1.

The *M. sexta* serpin-1 gene contains twelve alternate forms of exon 9, each of which encodes a different reactive center loop (18, 20). The serpin variants generated by alternative splicing differ in their inhibitory selectivities (21). To test whether the serpin-3 gene might have
a similar structure, we used two serpin-3-specific primers corresponding to the 3’ end of exon 8 and the 5’ end of exon 10 in the serpin-1 gene for PCR amplification of *M. sexta* genomic DNA (Fig. 1). The sequence of the amplified DNA fragment contained a 339 bp intron between nucleotides 1343 and 1344 of the serpin-3 cDNA sequence. Although this intron is located at the same position as the intron between exon 8 and exon 9A of the serpin-1 gene, serpin-3 gene does not contain any alternate exons in this region.

*Sequence comparisons*—Serpin-3 was most similar in amino acid sequence to a bacteria-induced serpin from another lepidopteran insect, *Hyphantria cunea* (39), with 60.3% identity. The second most similar sequence was *Drosophila* serpin 27A (40, 41), which shares 39.6% identity with serpin-3. Serpin-3 is only 29.8% identical to *M. sexta* serpin-1. A multiple sequence alignment of these insect serpins indicates that *M. sexta* serpin-3 and *H. cunea* serpin contain an amino-terminal extension of 45-50 amino acid residues that is absent from *M. sexta* serpin-1 and most other members of the serpin superfamily (Fig. 2). *Drosophila* serpin 27A has a shorter amino-terminal extension of ~30 residues. The putative reactive center loop of serpin-3 is from Gly$^{374}$ to Tyr$^{397}$, including Asn-Lys-Phe-Gly at the predicted P2 to P2’ positions. The same sequence is present in the *H. cunea* serpin and *Drosophila* serpin 27A. With a Lys residue as its putative P1 residue, serpin-3 probably inhibits serine proteinases with trypsin-like specificity.

*Purification of Recombinant Serpin-3 Protein from E. coli and insect cells*—We expressed recombinant serpin-3 with an amino-terminal hexahistidine tag in *E. coli* and purified it by nickel affinity chromatography under denaturing conditions (Fig. 3A). While the purified serpin-3 was used as an antigen for producing a polyclonal rabbit antiserum, it was inactive in
the inhibition assays even after renaturation (data not shown). In order to characterize and study
the function of serpin-3, we expressed it in insect cells using a baculovirus system. Serpin-3 was
secreted into the cell culture medium utilizing its own signal peptide. Concentrated cell culture
supernatant was separated by anion exchange chromatography on a Q-Sepharose column, and
serpin-3 eluted at ~150 mM NaCl at pH 7.5. The purified serpin-3 from the baculovirus
expression system (Fig. 3B) was used for characterization and functional analysis in later
experiments. It has an apparent molecular mass of 56.8 kDa as estimated by SDS-PAGE, which
is larger than the value calculated from the deduced amino acid sequence (48.9 kDa). This
difference may be a result of glycosylation. The first five amino acid residues of the purified
serpin-3 were determined to be Asp-Asp-Val-Asp-Pro, confirming the prediction that the signal
peptide ends at residue 20 (Fig. 1).

Serpin-3 mRNA and protein expression--In Northern blot analysis, a 2.5 kb serpin-3
mRNA was detected in RNA from fat body of insects that were challenged by bacterial injection
(Fig. 4A). A band at the same position could also be detected in RNA from control fat body and
hemocytes after very long autoradiography exposure (data not shown). The time course of
serpin-3 mRNA accumulation in response to bacterial challenge was examined by RT-PCR (Fig.
4B). The serpin-3 mRNA level began to increase by 1 h after injection of bacteria, reached a
maximum at 6-8 h, and remained elevated to 24 h.

We examined the induction pattern of serpin-3 protein in hemolymph by immunoblotting
(Fig. 5). The constitutive serpin-3 concentration was 5-12 µg/ml in naive larvae. In saline-
injected larvae, serpin-3 concentration was maintained at this basal level. The microorganisms
tested, including Gram-positive bacteria (M. luteus), Gram-negative bacteria (E. coli) and yeast
S. cerevisiae), stimulated similar changes in serpin-3 concentration. In larvae injected with yeast or E. coli, the serpin-3 protein level decreased during the first 2 h and then increased rapidly, whereas in the M. luteus-injected larvae, the protein level decreased and remained at a low level until 12 h after injection. The immediate decrease in serpin-3 concentration after microbial injection may reflect the use of constitutively expressed serpin-3 before new protein was synthesized to compensate the loss. Serpin-3 concentration reached a maximum at around 32 h post infection, with 78 µg/ml for yeast-treated larvae, 54 µg/ml for E. coli-treated larvae, and 29 µg/ml for M. luteus-treated larvae.

Inhibition of prophenoloxidase activation—The proPO activation cascade is a proteolytic pathway in insect hemolymph, which is triggered by microbial cell wall components. To investigate the physiological function of serpin-3, we tested its effect on proPO activation. The 0-50% ammonium sulfate fraction of M. sexta larval hemolymph, containing all the necessary components for proPO activation (21), was activated by incubation with M. luteus. Serpin-3 inhibited the activation of proPO by 90% at 61 ng/µl and 50% at 17 ng/µl (Fig. 6). This result indicates that serpin-3 inhibits at least one proteinase of the proPO cascade.

Inhibition of prophenoloxidase activating proteinases—We tested whether inhibition of PAPs by serpin-3 may explain the observed decrease in proPO activation. Recombinant M. sexta PAP-1 or PAP-3 were incubated with recombinant serpin-3 at different enzyme/inhibitor molar ratios. The residual amidase activity decreased linearly as serpin-3 concentration increased, and complete inhibition occurred at a serpin/enzyme ratio of 1.6 for PAP-1 and 1.9 for PAP-3 (Fig. 7). We determined the second-order association rate constants (kassoc) for inhibition of PAP-1 and PAP-3 by serpin-3 as an indicator of inhibitory selectivity. Serpin-3 had a kassoc of 7.5×10^5
M$^{-1}$ s$^{-1}$ for PAP-1 and 6.9×10$^5$ M$^{-1}$ s$^{-1}$ for PAP-3. Thus, serpin-3 inhibits PAP-1 and PAP-3 at rates comparable to those observed for other serpins in mammals and arthropods acting on their physiological target proteinases (Table 1).

In the inhibition reaction between a serpin and a susceptible proteinase, an inhibitor/enzyme complex is formed that is stable in SDS. Such complexes were detected when we incubated serpin-3 with either PAP-1 or PAP-3 (Fig. 8). The interaction of serpin-3 and PAP-1 resulted in the formation of a ~90 kDa complex under reducing conditions, which was absent from the controls lanes with only PAP-1 or serpin-3. This complex was recognized by both serpin-3 antibody and PAP-1 antibody (Fig. 8A and B), indicating that it was composed of these two proteins. The combined apparent molecular mass of serpin-3 (residues 1-388) (51.5 kDa) and the PAP-1 catalytic domain (34 kDa) is close to the size of the complex (~90 kDa). When the molar ratio of PAP-1 to serpin-3 was increased from 1:1 to 2:1, more of the serpin-3 was converted to the complex form (Fig. 8B, lanes 2 and 3). A band corresponding to a lower M$_r$ protein (51.5 kDa) was also detected by serpin antibody. Since this band was not present in the control of serpin-3 alone, it probably represents the cleaved serpin without the 5.3 kDa carboxyl-terminus (residues 389-435). This is consistent with the observation that more PAP-1 led to more of the cleaved form of serpin-3. The complex formation was blocked when an irreversible serine proteinase inhibitor, DFP, was preincubated with PAP-1 before addition of serpin-3 to the reaction mixture (Fig. 8B, lane 4). This result indicates that active PAP-1 is required for forming the complex. PAP-1 is composed of an amino-terminal clip domain and a carboxyl-terminal catalytic domain, which are linked by an interchain disulfide bond (7). Under reducing conditions, the clip domain is separated from the catalytic domain. The intensity of the
clip domain band recognized by PAP-1 antibodies was similar in each sample (Fig. 9A), whereas the catalytic domain band became much weaker after reaction with serpin-3, further indicating that linkage between serpin-3 and the active site of the catalytic domain resulted in complex formation. Similar results were obtained for the inhibition of PAP-3 by serpin-3. A complex at approximately 90 kDa was formed between serpin-3 (51.5 kDa) and the PAP-3 catalytic domain (39 kDa) (Fig. 8, panels C and D).

Next, we tested whether serpin-3 can inhibit and form a stable complex with the PAPs under more physiological conditions. Hemolymph collected from naive *M. sexta* larvae was incubated for 5 min with *M. luteus* to stimulate proPO activation. Serpin-3, constitutively present at a basal level, may inhibit the activated PAPs and form serpin-proteinase complexes during this process. After 5 min, remaining active serine proteinases were then inactivated with 5 mM DFP. These samples were applied to an immunoaffinity column made with serpin-3 antibody to isolate any complex formed (as well as free serpin-3). The proteins that bound to the affinity column were analyzed by immunoblotting using either PAP or serpin-3 antibodies. Immunoblot analysis using PAP-1 antibodies revealed a ~90 kDa band, which is present at a much lower intensity in the control, untreated sample (Fig. 9A). Similarly, PAP-3 antibodies detected a band at about the same size in *M. luteus*-treated hemolymph, which was hardly detected in the control (Fig. 9B). This result demonstrated that in plasma, the activated PAP-1 and PAP-3 resulting from the bacteria-triggered activation of the PPO cascade can be inhibited by serpin-3, with consequent formation of a stable serpin-3-PAP complex. The complex in naive hemolymph may be due to a trace amount of enzyme that was spontaneously activated during hemolymph collection. The same samples were also examined using serpin-3 antibodies, which
revealed two bands at the size range of serpin-3-proteinase complexes (Fig. 9C). We have not yet determined from this experiment which band represents which proteinase complex.

DISCUSSION

In the present study, we characterized the newly discovered serpin-3 from *M. sexta* hemolymph. It is constitutively expressed at a low level in fat body and secreted into hemolymph. After injection of bacteria or yeast, the serpin-3 mRNA and protein levels increase dramatically. We found that serpin-3 efficiently inhibits PAP-1 and PAP-3 and forms covalent complexes with the purified enzymes and with endogenous PAP-1 and PAP-3 in plasma. The *in vivo* half-life of PAP-1 or PAP-3, calculated as $1/k_{assoc} \times [I]$, is 6-15s in naive larval hemolymph and 0.9-2.8s in induced larval hemolymph, suggesting that serpin-3 could rapidly inhibit PAP-1 and PAP-3 under physiological conditions. Taken together, these data provide strong evidence that PAP-1 and PAP-3 are natural targets of serpin-3 and that serpin-3 blocks the proPO activation cascade by inhibiting the PAPs specifically and rapidly. Preliminary experiments suggest that PAP-2 may also be regulated by serpin-3 (Jiang *et al.*, unpublished results). Several insect inhibitors that block the proPO cascade have been reported (21, 39-41, 46, 47), but the natural proteinase targets of these inhibitors have not been assigned. To our knowledge, this is the first report to provide direct evidence that an insect serpin can block the proPO activation cascade by specifically and rapidly inhibiting PAPs from the same species.

It is not surprising that PAP-1 and PAP-3 share a regulator because the enzymes are similar and they cleave the same substrates. They have an overall amino acid sequence identity of 42%, and many key residues are conserved. PAP-2 and PAP-3 cleave the two subunits of
proPO at Arg\textsuperscript{51} within the sequence Asn-Arg-Phe-Gly (7, 8, 37, 48). Serpin-3 contains Asn-Lys-Phe-Gly at its predicted P2-P2' position, which is strikingly similar to the proPO cleavage site. This sequence similarity may explain the structural basis for the highly specific and efficient binding between the PAPs and serpin-3.

The cleavage site of proPOs from different insect species and a crayfish are highly conserved with an occasional substitution of (P2) Asn to Ser or Thr and (P2') Gly to Ser (39). Serpins with a P2-P2' sequence similar to the proPO activation site, perhaps homologs of serpin-3, may be present in other insect species to regulate proPO activation. Indeed, an immune-responsive \textit{H. cunea} serpin, which is 60\% identical to serpin-3, was shown to inhibit proPO activation (39). Its target protease was not identified. \textit{Drosophila} serpin 27A, which is 40\% identical to serpin-3 and which has the same P2-P2' residues, is also immune inducible and inhibits activation of proPO (40, 41). \textit{M. sexta} serpin-3, \textit{H. cunea} serpin, and \textit{Drosophila} serpin 27A all have an extended amino-terminal sequence that is not present in most other serpins. Spn27A mutants have higher PO activity than wild type flies, they develop melanized tissues, and they react to injury with uncontrolled melanization (40, 41). The natural target of Spn27A is unknown, but the serpin inhibits a prophenoloxidase activating enzyme (PPAE) purified from the beetle \textit{Holotrichia diomphalia}, suggesting that Spn27A may inhibit a \textit{Drosophila} PPAE (40). \textit{M. sexta} serpin-1J is another serpin that blocks proPO activation (21) and forms complexes with PAP-1, -2, and -3 (Wang \textit{et al.}, unpublished results; 9). The reactive site of serpin-1J is DRCC, which is less similar to the proPO cleavage sequence than the serpin-3 P2-P2' sequence. Once kinetic data for serpin-1J inhibition of the PAPs are available, we can compare the inhibitory efficiency and specificity of serpin-1J and serpin-3.
Serpin-3 is an immune-responsive protein. It is constitutively expressed but rapidly up-regulated at the mRNA level after bacterial injection. PAP-1 and PAP-3 are also constitutively expressed at a low level and are immune inducible (9, 32). The similar induction patterns among serpin-3 and the PAPs are consistent with regulation of the PAPs by serpin-3. In healthy larvae, serpin-3 may function as a scavenger to inactivate any spontaneously activated PAPs to avoid unnecessary melanization, and, thus, protect the host from the cytotoxic molecules produced during this process. The small amount of complex formed between serpin-3 and PAP-1 and PAP-3 in naive larvae and the fast kinetics of the PAP-serpin-3 reactions support this hypothesis. In infected larvae, inhibition of the PAPs by serpin-3 may be necessary to prevent systemic melanization. We expect that serpin-3 acts to limit the duration of the melanization reaction and restrict its location to the infection site.
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Footnotes

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The nucleotide sequences reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession numbers AF413064 and AF413065.

The abbreviations used are: PO, phenoloxidase; proPO, prophenoloxidase; PAP, prophenoloxidase-activating proteinase; PPAE, prophenoloxidase-activating enzyme; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; IEARpNa, acetyl-Ile-Glu-Ala-Arg-p-nitroanilide; RT, reverse transcriptase; PCR, polymerase chain reaction; RSL, reactive site loop; PBS, phosphate-buffered saline.
Figure Legends

Fig. 1  Nucleotide and amino acid sequence of *M. sexta* serpin-3. *A*, The deduced amino acid sequence is shown below the nucleotide sequence of serpin-3A (accession number AF413064). The one-letter codes are aligned with the second nucleotide of each codon. The 20-residue signal peptide is underlined and assigned negative numbers. Four potential *N*-linked glycosylation sites are marked with “*” after the Asn residues. The predicted P1 and P1’ residues are indicated. A polyadenylation signal (AATAAA, double underlined) is near the 3’-end of the cDNA. Two full-length clones were obtained with small differences in nucleotide sequence. Substitutions in the open reading frame of serpin-3B (accession number AF413065) are indicated with italic letters above the nucleotide sequence of serpin-3A. *B*, The nucleotide sequence of an intron (obtained by PCR amplification of genomic DNA) is located between nucleotides 1343 and 1344 of the cDNA (marked ). The two underlined nucleotide sequences in part A correspond to the primers for PCR amplification of the genomic fragment containing the intron.

Fig. 2  Sequence alignment of *M. sexta* serpin-3 with other insect serpins. The amino acid sequence of *M. sexta* serpin-3 (Ms spn-3) is aligned with *H. cunea* serpin (Hc spn), *Drosophila melanogaster* serpin 27A (Dm spn27A), and *M. sexta* serpin-1J (Ms spn-1J), excluding the signal peptides. The residues conserved in all four sequences are shaded. The predicted RSL of serpin-3 (P15-P9’) is underlined, and the putative P2-P2' site is indicated above the sequence.

Fig. 3  SDS-PAGE and immunoblot analysis of purified recombinant serpin-3. *A*, Serpin-3 expressed in *E. coli* and purified by nickel affinity chromatography. *B*, Serpin-3 produced in the
baculovirus-insect cell expression system. The recombinant protein in the cell culture medium was concentrated and purified as described under “Experimental Procedures”. Both A and B show Coomassie blue-stained gels. C. Antiserum raised against purified recombinant serpin-3, produced in E. coli, was used to detect the recombinant serpin-3 produced by Sf9 cells (lane 1), serpin-3 in naive hemolymph (lane 2) and in hemolymph (lane 3) collected from larvae at 24 h after injection with E. coli.

**Fig. 4 Expression and induction pattern of serpin-3 mRNA.** A. Northern blot analysis of serpin-3 mRNA expression. Twenty µg of total RNA isolated from fat body (F) or hemocytes (H) at 24 hr after injection with saline (C for control) or M. luteus (I for immunized) was separated by electrophoresis on a 1% formaldehyde agarose gel and transferred onto nylon membrane. A serpin-3 cDNA fragment was labeled with $^{32}$P to probe the RNA blot. To confirm equal loading, a duplicate blot was probed with $^{32}$P-labeled ribosomal protein S3 (rpS3) cDNA. B. RT-PCR analysis of serpin-3 mRNA induction pattern. Day 2, fifth instar larvae were injected with M. luteus or saline. Total RNA was isolated from fat body dissected at different times after the bacterial challenge (0.5-24 hr, four insects for each time). In the control (0), total RNA was obtained from fat body at 24 h after saline injection. RT-PCR was performed as described under “Experimental Procedures” using gene-specific primers for serpin-3 and rpS3 as test and internal control, respectively.

**Fig. 5 The induction pattern of serpin-3 protein in hemolymph.** Day 2, 5th instar larvae were injected with saline, Gram-positive bacteria (M. luteus), Gram-negative bacteria (E. coli), or
yeast (*S. cerevisiae*). Plasma samples were collected at different times after the injection for SDS-PAGE and immunoblot analysis. Serpin-3 concentration was determined by densitometry (32), using known amounts of purified recombinant serpin-3 as a standard. The error bars represent the standard error of the mean (n=4).

**Fig. 6** Inhibition of proPO activation by serpin-3. A proPO activation fraction of *M. sexta* hemolymph was incubated with purified recombinant serpin-3 at different concentrations (0-61 ng/µl). Activation of proPO was then triggered by adding *M. luteus* to a final concentration of 0.1 µg/µl. PO activity (mean±SD, n=3) was measured in a spectrophotometer after 10 min (37).

**Fig. 7** Stoichiometry for inhibition of *M. sexta* PAP-1 and PAP-3 by serpin-3. Purified recombinant serpin-3 was incubated with recombinant PAP-1 (*A*) or PAP-3 (*B*) at various molar ratios (0.4:1 to 4:1) for 10 min at room temperature. The residual amidase activity was then measured using an artificial substrate, IEARpNa (8). The intersection of a line (using the first 5 data points) extrapolated to the x-axis occurred at a molar ratio of 1.6:1 for PAP-1 and 1.9:1 for PAP-3.

**Fig. 8** In vitro complex formation between serpin-3 and PAPs. Purified recombinant serpin-3 was incubated with PAP-1 (*A, B*) or PAP-3 (*C, D*) for 10 min at room temperature. The mixtures were subjected to SDS-PAGE under reducing conditions, and the complex was detected by immunoblotting using antisera raised against either PAP (*A, C*) or serpin-3 (*B, D*). *A*, lane 1: PAP-1 only; lane 2: PAP-1 + serpin-3 at 1:1 molar ratio; lane 3: PAP-1 + serpin-3 at 2:1 molar.
ratio; lane 4: PAP-1 inhibited by preincubation with DFP before addition of serpin-3.  

**B**, lane 1: serpin-3 only; lanes 2-4: same as lanes 2-4 in A.  

**C**, same as A, except that PAP-1 was replaced by PAP-3.  

**D**, same as B, except that serpin-3 was incubated with PAP-3 instead and the lane for serpin-3 only is not shown.  
The first antibody used is specified under each panel.  Sizes of the Mr markers are indicated on the left of each blot.  

**Fig. 9 Complex formation between serpin-3 and PAPs in plasma.** Plasma samples from day 2, fifth instar larvae were incubated with 5 mM DFP immediately or with 100 µg/ml *M. luteus* for 5 min followed by DFP addition to stop the reaction.  
The serpin-3-proteinase complexes were isolated by separately passing the two reaction mixtures through serpin-3 antibody affinity columns. The proteins that bound to the column were eluted and subjected to immunoblot analysis using PAP-1 (A), PAP-3 (B), or serpin-3 (C) antibodies.  

Lane 1: no bacteria added; Lane 2: 5 min incubation with *M. luteus* before adding DFP.  
A band recognized by the PAP antisera was found to be protein A that eluted from the immunoaffinity support.
| serpin                | proteinase                 | $k_\text{ass} \ (\text{M}^{-1}\text{s}^{-1})$ | reference |
|-----------------------|----------------------------|---------------------------------|-----------|
| antithrombin III      | thrombin                   | $1.1 \times 10^4$               | 42        |
| PAI-1                 | tPA                        | $5.2 \times 10^6$               | 43        |
| protein C inhibitor   | acrosin                    | $2.4 \times 10^5$               | 44        |
| LICI-1                | *Limulus* factor C         | $2.5 \times 10^6$               | 45        |
| LICI-2                | *Limulus* factor C         | $7.1 \times 10^4$               | 45        |
| LICI-2                | *Limulus* clotting enzyme  | $4.3 \times 10^5$               | 45        |
| LICI-3                | *Limulus* factor G         | $3.9 \times 10^5$               | 45        |
| serpin-3              | PAP-1                      | $7.5 \times 10^5$               |           |
| serpin-3              | PAP-3                      | $6.9 \times 10^5$               |           |

PAI, plasminogen activator inhibitor; tPA, tissue-type plasminogen activator; LICI, *Limulus* intracellular coagulation inhibitor; PAP, proPO-activating proteinase.
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 7
Fig. 8
Fig. 9
Manduca sexta serpin-3 regulates prophenoloxidase activation in response to infection by inhibiting prophenoloxidase-activating proteinases
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