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

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ABSTRACT

Serpins are a superfamily of proteins, most of which inhibit cognate serine proteases by forming inactive acyl-enzyme complexes. In the tobacco hornworm *Manduca sexta*, serpin-1, -3 through -7 negatively regulate a hemolymph serine protease system that activates precursors of the serine protease homologs (SPHs), phenoloxidases (POs), Spätzles, and other cytokines. Here we report the cloning and characterization of *M. sexta* serpin-9 and -13. Serpin-9, a 402-residue protein most similar to *Drosophila* Spn77Ba, has R^{366} at the P1 position right before the cleavage site; Serpin-13, a 444-residue ortholog of *Drosophila* Spn28Dc, is longer than the other seven serpins and has R^{410} as the P1 residue. Both serpins are mainly produced in fat body and secreted into plasma to function. While their mRNA and protein levels were not up-regulated upon immune challenge, they blocked protease activities and affected proPO activation in hemolymph. Serpin-9 inhibited human neutrophil elastase, cathepsin G, trypsin, and chymotrypsin to different extents; serpin-13 reduced trypsin activity to approximately 10% at a molar ratio of 4:1 (serpin: enzyme). Serpin-9 was cleaved at Arg^{366} by the enzymes with different specificity, but serpin-13 had four P1 sites (Arg^{410} for trypsin-like proteases, Gly^{406} and Ala^{409} for the elastase and Thr^{404} for cathepsin G). Supplementation of induced cell-free hemolymph (IP, P for plasma) with recombinant serpin-9 did not noticeably affect proPO activation, but slightly reduced the PO activity increase after 0–50% ammonium sulfate fraction of the IP had been elicited by bacteria. In comparison, addition of recombinant serpin-13 significantly inhibited proPO activation in IP and the suppression was stronger in the fraction of IP. Serpin-9- and -13-containing protein complexes were isolated from IP using their antibodies. Hemolymph protease-1 precursor (proHP1), HP6 and HP8 were found to be associated with serpin-9, whereas proHP1, HP2 and HP6 were pulled downed with serpin-13. These results indicate that both serpins regulate immune proteases in hemolymph of *M. sexta* larvae.

1. Introduction

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

A framework of immune serine protease system has been

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

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

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

2. Materials and methods

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

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Each of day 2, 5th instar larvae was injected with a mixture of *Escherichia coli* (1.3×10^7 cells), *Micrococcus luteus* (13 μg), and curdlan (13 μg) in 20 μl H_2O. Induced hemolymph was collected from cut prolegs of the larvae 24 h later and centrifuged at 5000×*g* for 5 min to remove hemocytes and obtain induced plasma (IP). The plasma samples were aliquoted and stored at −80 °C for proPO activation, serpin-protease complex isolation, and other experiments. Similarly, control plasma was prepared from day 3, 5th instar naïve larvae. To increase sensitivity of the proPO activation assay, a known volume of the induced hemolymph was treated with equal volume of 100% saturated ammonium sulfate to remove most of the negative regulators (*e.g.* serpins, PO inhibitors) while keeping the proPO activation system intact in the $(NH_4)_2SO_4$ fraction. After centrifugation, the pellet was resuspended in the same volume of 20 mM NaCl, 20 mM Tris-HCl, pH 7.5, aliquoted, and stored at −80 °C as "0–50% $(NH_4)_2SO_4$ fractionated IP" or "0–50 IP".

Total RNA samples were isolated from hemocytes (H) and fat body (F) of the induced (I) and naïve or control (N or C) larvae using Trizol Reagent (Life Technologies). Integument, Malpighian tubule, midgut, muscle, nerve tissue, salivary gland, and trachea were dissected from 5 to 10 of day 3, 5th naïve larvae for RNA extraction. In the developmental series, fat body and cell-free hemolymph were collected from naïve larvae and pupae at various life stages (3 to 6 insects per stage) for RNA isolation and immunoblot analysis, respectively. For cDNA synthesis, the total RNA samples (1 μg each) were incubated with 1×iScript Reverse Transcription Supermix (Bio-Rad) in a 10 μl reaction at 25 °C for 5 min, 42 °C for 60 min, and heated at 95 °C for 5 min to inactivate the enzyme.

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

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

2.3. Multiple sequence alignment and phylogenetic analysis

Amino acid sequences of the serpins of *M. sexta, Bombyx mori, Anopheles gambiae, and Drosophila melanogaster* were retrieved from NCBI and aligned using MUSCLE using the following parameters: refining alignment, gap opening penalty = −2.9, gap extension penalty = 0, hydrophobicity multiplier = 1.2, maximum iterations = 100, clustering method (for iterations 1 and 2) = UPGMB, and maximum diagonal length = 24. The aligned sequences were converted to NEXUS format by MEGA (Kumar et al., 2016), and phylogenetic analyses were conducted using MrBayes v3.2.6 (Ronquist et al., 2012) under the default model with the setting "nchains = 12". MCMC (Markov chain Monte Carlo) analyses were terminated after the standard deviations of two independent analyses were <0.01. FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/) was used to display the phylogenetic trees.

2.4. Recombinant expression and polyclonal antibody generation

The serpin-9 cDNA was used as a template for PCR amplification