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A positive feedback mechanism in the *Manduca sexta* prophenoloxidase activation system

Yang Wang, Haobo Jiang *

Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, USA

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ABSTRACT

In *Manduca sexta*, pathogen recognition triggers a branched serine proteinase cascade which generates active phenoloxidase (PO) in the presence of a proPO-activating proteinase (PAP) and two noncatalytic serine proteinase homologs (SPHs). PO then catalyzes the production of reactive compounds for microbe killing, wound healing, and melanin formation. In this study, we discovered that a minute amount of PAP1 (a final component of the proteinase pathway) caused a remarkable increase in PO activity in plasma from naïve larvae, which was significantly higher than that from the same amounts of PAP1, proPO and SPHs incubated *in vitro*. The enhanced proPO activation concurred with the proteolytic activation of HP6, HP8, PAP1, SPH1, SPH2 and PO precursors. PAP1 cleaved proSPH2 to yield bands with mobility identical to SPH2 generated *in vivo*. PAP1 partially hydrolyzed proHP6 and proHP8 at a bond amino-terminal to the one cut in the PAP1-added plasma. PAP1 did not directly activate proPAP1. These results suggest that a self-reinforcing mechanism is built into the proPO activation system and other plasma proteins are required for cleaving proHP6 and proHP8 at the correct site to strengthen the defense response, perhaps in the early stage of the pathway activation.

1. Introduction

Serine proteinases in vertebrates and invertebrates constitute pathways to mediate the innate immunity upon tissue damage or pathogen invasion (Krem and Di Cera, 2002; Jiang and Kanost, 2000). The blood coagulation system in mammals is an example of such extracellular proteinase cascades. Positive and negative feedback loops exist in the system to control its potency and duration. Thrombin activates its upstream coagulation factors via limited proteolysis in the early stage of blood clotting (Dahlback and Villoutreix, 2005). As the coagulation proceeds, thrombin binds to thrombomodulin and becomes an anticoagulant by inactivating its upstream enzymes at different sites. Serpins regulate blood coagulation by forming irreversible complexes with the clotting factors. Analogous proteinase cascades in arthropod hemolymph mediate defense responses, and serpins negatively modulate these processes. While proteinase-mediated positive feedback occurs during the establishment of dorsoventral axis in *Drosophila* (Dissing et al., 2001), it is unclear whether or not such mechanism exists in arthropod immune proteinase pathways (e.g. prophenoloxidase (proPO) activation system).

Insect phenoloxidases (POs) produce quinones and other reactive compounds for melanin formation, protein crosslinking and microbe killing (Ashida and Brey, 1998; Cerenius and Söderhäll, 2004; Nappi and Christensen, 2005; Zhao et al., 2007). POs are synthesized as inactive precursors and proteolytically activated by proPO activating proteinases (PAPs) (Satoh et al., 1999; Jiang et al., 1998, 2003a, b; Lee et al., 1998; Tang et al., 2006). In some insects, proPO activation requires the presence of one or two noncatalytic SPHs (Kwon et al., 2000; Yu et al., 2003). The PAPs and SPHs, containing a regulatory clip domain at the amino terminus, are activated in a branched serine proteinase cascade (Kim et al., 2002). This cascade is initiated upon recognition of pathogen surface molecules by specific binding proteins. *Manduca sexta* hemolymph proteinase 14 precursor (proHP14) interacts with Gram-positive bacteria or fungi, self-activates, and cleaves proHP21 (Ji et al., 2004; Wang and Jiang, 2006). Active HP21 produces PAP2 and PAP3 (Gorman et al., 2007; Wang and Jiang, 2007), which generate active PO in the presence of SPH1 and SPH2. The SPH precursors are not active as a cofactor for the PAPs until a specific cleavage occurs (Lu and Jiang, 2008). To date, the activating proteinase(s) for proSPHs are not known in the tobacco hornworm. Neither is it clear whether proSPH activator(s) relate to the branch that leads to proPAP2 and proPAP3 activation.

We have isolated 27 hemolymph proteinase cDNAs from *M. sexta* and prepared polyclonal antisera to the recombinant

* Corresponding author. Tel.: +1 405 744 9400; fax: +1 405 744 6039.
E-mail addresses: haobo@okstate.edu, haobo.jiang@okstate.edu (H. Jiang).

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proteins (Jiang et al., 2005). Using the sequence information and molecular probes, we were able to identify HPs that are involved in the innate immune responses (Tong and Kanost, 2005; Tong et al., 2005; Zou and Jiang, 2005). Some of these enzymes form covalent complexes with *M. sexta* serpin-4 -5 and -6 which inhibit HP1, HP6, HP8, HP21 and PAPs. In this paper, we report an unexpected finding that by adding a small amount of PAP-1 to the larval plasma proPO activation was drastically enhanced. Using antisera against the HPs and SPHs, we detected changes in the cell-free hemolymph which explain the large increase in PO activity. Based on results from the immunoblot analysis and *in vitro* activation tests, we propose a positive feedback mechanism which represents a new layer of regulation during proPO activation.

2. Methods and materials

2.1. Insect rearing, hemolymph collection, protein preparation and activity assays

M. sexta eggs were purchased from Carolina Biological Supply and the larvae were reared on an artificial diet (Dunn and Drake, 1983). Hemolymph samples collected from cut prolegs of the day 3, fifth instar naïve larvae were centrifuged at 14,000*g* for 5 min to remove the hemocytes. The plasma sample was stored at −80 °C as 20 μl aliquots. ProPO and PAP1 were purified from the larval hemolymph and cuticle extract, respectively (Jiang et al., 1997; Gupta et al., 2005). The precursors of SPH1, SPH2 and PAP1 were prepared previously (Lu and Jiang, 2008; Wang et al., 2001). PO activity and acetyl-Ile-Glu-Ala-*p*-nitroanilide (IEAR*p*NA) hydrolysis were measured according to Jiang et al. (2003a), with one unit defined as the amount of enzyme causing 0.001 absorbance increase in 1 min .

2.2. Enhancement of proPO activation in plasma from naïve larvae

Cell-free hemolymph (1.0 μl) was incubated on ice for 60 min with buffer A (20 mM Tris–HCl, pH 8.0, 19 μl), purified SPHs (1.0 μl, 20 ng/μl and 18 μl buffer A), or PAP1 (1.0 μl, 20 ng/μl and 18 μl buffer A). As controls, proPO alone (1.0 μl, 100 ng/μl and 19 μl buffer A) and proPO activation mixture (100 ng proPO, 20 ng SPHs, 20 ng PAP1, and 17 μl buffer A) were incubated under the same condition prior to PO activity measurement.

2.3. Immunoblot analysis of proPO, proSPH and proHP proteolysis in the plasma

Plasma (30 μl) from naïve larvae was incubated at room temperature for 15 min with buffer A (165 μl) or PAP1 (15 μl, 20 ng/μl and 150 μl buffer A) in the presence of 0.01% 1-phenyl-2-thiourea. After being treated with SDS sample buffer containing 2-mercaptoethanol, the samples (equivalent to 1.5 μl plasma) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Following electrotransfer and blocking, the protein blots were individually reacted with 1:2000 diluted polyclonal antisera against proPO, PAP1, PAP2, PAP3, SPH1, SPH2, HP1, HP2, HP5, HP6, HP8, HP14, HP16, HP17, HP19 and HP21. The antigen–antibody complexes were visualized by color precipitation catalyzed by alkaline phosphatase conjugated to goat-anti-rabbit IgG (Bio-Rad).

2.4. Construction of recombinant baculoviruses for proHP6 and proHP8 production

M. sexta HP6 and HP8 cDNAs (Jiang et al., 2005) were amplified by PCR using specific primer pairs: j510 (5′-TTAGGATCCATGTGGTTAATGGTG) and j500 (5′-AGTCTCGAGATTAGGCCAAACAATAC) for HP6; j511 (5′-TTGGGATCCGTGTGTGAACTAG) and j501 (5′-GCACTCGAGAGGTCGTAACTTTGA) for HP8. The thermal cycling conditions were: 94 °C, 1 min; 35 cycles of 94 °C for 30 s, 40 °C for 30 s and 68 °C for 2 min; 68 °C for 3 min. The PCR products (1.08 and 1.12 kb) were digested with *Bam*HI completely and *Xho*I partially due to an internal *Xho*I site in the cDNA sequences. Following agarose gel electrophoresis, the cDNA fragments at the right size were inserted to the same sites in pFH6 (Ji et al., 2003). The resulting plasmids (proHP6/pFH6 and proHP8/pFH6) were sequenced to verify the correct insertion. *In vivo* transposition of the expression cassette, selection of bacterial colonies carrying the recombinant bacmid, and isolation of bacmid DNA were performed according to manufacturer's protocols (Invitrogen Life Technologies). The initial viral stocks (V_0) were obtained by transfecting *Spodoptera frugiperda Sf*21 cells with a bacmid DNA-CellFECTIN mixture, and their titers were improved through serial infection. The V_5 viral stock, containing the highest level of baculovirus, was stored at −70 °C for further experiments. Expression conditions were optimized according to Ji et al. (2003).

2.5. Expression of recombinant proHP6 and proHP8 in insect cells

Sf 9 cells (at 2.4×10^6 cells/ml) in 150 ml of insect serum-free medium (Invitrogen Life Technologies) supplemented with 0.02 mM *p*-aminobenzamidine (*p*Abz) were infected with the proHP6 baculovirus stock at a multiplicity of infection of ten and grown at 27 °C for 75 h with agitation at 100 rpm. The expression of proHP8 was performed under the same conditions except that *Sf*21 cells (400 ml) were infected by the proHP8 viral stock for 96 h.

2.6. Isolation of recombinant proHP6 and HP6

After the cells were removed by centrifugation at 5000 *g* for 10 min, pH of the conditioned medium was adjusted to 8.4 using 100 ml 75 mM NaOH containing 2 mM *p*Abz. The cell debris and fine particles were spun down by centrifugation at 10,000*g*, and the supernatant was applied at a flow rate of 1 ml/min onto a Q-Sepharose FF column (1.5 cm i.d. × 11 cm) equilibrated with buffer B (20 mM Tris–HCl, pH 8.3, 0.01% Tween-20, 1 mM *p*Abz). After washing with 100 ml buffer B, bound proteins were eluted from the column with a linear gradient of 0–0.5 M NaCl in 100 ml buffer B and 0.5 M NaCl in 40 ml buffer B. The proHP6 fractions (3.4 ml/tube) were combined and mixed with 0.5 ml Ni^{2+}-nitrilotriacetic acid (NTA) agarose at 4 °C for 1 h with gentle agitation. The suspension was packed to an empty column, and the resin was washed with 10 ml buffer C (50 mM Tris–HCl, pH 8.0, 0.01% Tween-20, 100 mM NaCl) containing 10 mM imidazole and eluted with 250 mM imidazole in buffer C (0.5 ml/fraction). The HP6 fractions from the Q-column were affinity purified under the same conditions.

2.7. Partial purification of recombinant proHP8 and HP8

HP8 was partially purified from 200 ml conditioned medium on Q-Sepharose and Ni^{2+}-NTA agarose columns as described above. ProHP8 was purified from the cell lysate from 400 ml culture: infected cells harvested by centrifugation at 5000*g* for 10 min were treated with 40 ml of 50 mM sodium phosphate, pH