Published in final edited form as:

Insect Biochem Mol Biol. 2016 September ; 76: 1–10. doi:10.1016/j.ibmb.2016.06.009.

In search of a function of *Manduca sexta* hemolymph protease-1 in the innate immune system

Fan Yang, Yang Wang, Yan He, and Haobo Jiang

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

Abstract

Extracellular serine protease cascades mediate immune signaling and responses in insects. In the tobacco hornworm *Manduca sexta*, nearly 30 serine proteases (SPs) and their homologs SPHs) are cloned from hemocytes and fat body. Some of them participate in prophenoloxidase proPO) activation and proSpätzle processing. Here we report the cDNA cloning of hemolymph protease-1b (HP1b), which is 90% identical and 95% similar to HP1a (formerly HP1). The HP1a and HP1b mRNA levels in hemocytes was down- and up-regulated after an immune challenge, respectively. Quantitative real-time polymerase chain reactions revealed their tissue-specific and development-dependent expression, mostly in hemocytes of the feeding larvae. We isolated HP1 precursor (proHP1) from larval hemolymph and observed micro-heterogeneity caused by *N*-linked glycosylation. Supplementation of the purified proHP1 to plasma samples from naïve larvae or induced ones injected with bacteria caused a small PO activity increase, much lower than those elicited by recombinant proHP1a/b, but no proteolytic cleavage was detected in the zymogens. Incubation of proHP1a/b or their catalytic domains with a cationic detergent, cetylpyridinium chloride, induced an amidase activity that hydrolyzed LDLH-*p*-nitroanilide. Since LDLH corresponds to the P4–P1 region before the proteolytic activation site of proHP6, we propose that the active but uncleaved proHP1 may cut proHP6 to generate HP6 that in turn activates proPAP1 and proHP8. The catalytic domain of HP1a/b, which by itself does not activate purified proHP6 or hydrolyze LDLH-*p*-nitroanilide, somehow generated active HP6, HP8, PAP1 and PO in plasma. Together, these results indicate that proHP1 participates in the proPO activation system, although detailed mechanism needs further exploration.

Graphical Abstract

Send correspondence to: Haobo Jiang, Department of Entomology and Plant Pathology, Oklahoma State University, Stillwater, OK 74078, Telephone: (405)-744-9400, Fax: (405)-744-6039, haobo.jiang@okstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

clip domain; insect immunity; phenoloxidase; hemolymph protein; enzyme cascade; zymogen activation

1. Introduction

Over thirty serine proteases (SPs) in human plasma mediate blood coagulation, fibrinolysis, and complement activation (O'Brien and McVey, 1993). They are normally present as inactive zymogens in the circulation and are sequentially activated upon recognition of aberrant tissues or microbial surface molecules. Through specific interaction and limited proteolysis, a localized reaction is initiated within minutes to stop bleeding, dismantle clots, or attack invading microbes. The reaction extent is regulated by serpins that form acyl-complexes with their target proteases (Gettins, 2002). Complex SP pathways have also evolved in arthropods for hemolymph clotting, cytokine processing, and prophenoloxidase (proPO) activation (Iwanaga et al., 1998; Jiang et al., 2010; Park et al., 2010; Kurata, 2010). Some pathway members comprise an amino-terminal clip domain and a carboxyl-terminal catalytic domain (Kanost and Jiang, 2015). Cleaved clip-domain serine protease homologs (SPHs, or SPs whose catalytic Ser substituted by Gly) are also involved in insect proPO activation (Kwon et al., 2000; Yu et al., 2003). Serpins regulate the SP-SPH system so that the cascade reaction occurs as a local, transient response.

There are 193 SP/SPH genes in the genome of *Manduca sexta*, 68 encoding proteins that are likely involved in digestion of dietary proteins (Cao et al., 2015). The remaining 125 genes are expressed in other tissues at different life stages. In the proteomic analysis of larval hemolymph, we identified 36 SPs and SPHs, nine of which are known to be components of the SP-SPH system (Zhang et al., 2014; He et al., 2016). They are: HP14 (HP for hemolymph protease), HP21, HP6, HP8, proPO activating proteases (PAP1–3), SPH1, and SPH2 (Jiang et al., 2010; Wang et al., 2014). We isolated SP and SPH cDNAs (or genes) from hemocytes or fat body for HP1–4, PAP1–3, HP5–24, SPH1, and SPH2 (Jiang et al., 1998, 1999, 2003a, 2003b and 2005; Wang et al., 2006; Yu et al., 2003). However, biochemical functions of the other 27 SP-related proteins in hemolymph are mostly unknown. For instance, HP6 activates proPAP1 and proHP8 to stimulate melanization and antimicrobial peptide (AMP) synthesis, respectively (An et al., 2009 and 2010), but it is unclear how proHP6 is activated by an HP or how this unknown HP is activated upon recognition of invading microbes. In fact, despite all the efforts, we do not even understand the molecular function of HP1, the first clip-domain SP cloned from *M. sexta*.

M. sexta HP1 is expressed in hemocytes (granulocytes and oenocytoids) but not in fat body of feeding larvae, its protein product (*c.a.* 50 kDa) is mostly present in plasma (Jiang et al., 1999). We observed small sequence variations in the HP1 cDNA fragments and, based on the cDNA clone obtained by library screening, HP1 (now HP1a) is predicted to be a trypsin-like SP activated upon cleavage between AQGR and VFGS. The active HP1 should have an N-terminal regulatory clip domain and a C-terminal catalytic domain, linked by an interchain disulfide bond. However, we have not observed this type of HP1 that dissociates into two chains in the presence of a reducing agent (*e.g.* dithiothreitol or DTT). Here we report some of the results obtained over the years regarding HP1's role in melanization and how it may be involved in proHP6 activation. In this paper, we describe the cDNA cloning of HP1b, expression profiles of HP1a and HP1b, purification and functional analyses of proHP1a, as well as possible mechanisms of proHP1 activation.

2. Materials and methods

2.1. Insect rearing, immune challenge, and cDNA synthesis

M. sexta eggs were purchased from Carolina Biological Supply and the 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* (2×10^7 cells), *Micrococcus luteus* (20 µg) (Sigma-Aldrich), and curdlan (20 µg, insoluble β -1,3-glucan from *Alcaligenes faecalis*) (Sigma-Aldrich) in 30 µl H₂O. Hemolymph samples were collected from cut prolegs of three larvae 24 h later and centrifuged at 5,000*g* for 5 min to remove hemocytes. Total RNA samples were isolated from induced hemocytes (IH) and fat body (IF) using TRIZOL Reagent (Life Technologies, Inc). Control hemocyte (CH) and fat body (CF) RNA samples were prepared from three day 3, 5th instar naïve larvae. Similarly, integument, midgut, Malpighian tubule, muscle, nerve tissue, salivary gland, and trachea were dissected from 5–10 day 3, 5th naïve larvae for RNA preparation. In the developmental series, hemocytes and cell-free hemolymph were prepared from larvae and pupae at different life stages (3–6 insects per stage) for RNA isolation and immunoblot analysis, respectively. For cDNA synthesis, these 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 30 min, and heated at 85°C for 5 min to inactivate the enzyme.

2.2. cDNA cloning and structural features of *M. sexta* HP1b

The 33 HP1 cDNA clones (Jiang et al., 2005) were analyzed to identify possible causes of the sequence variations in HP1 cDNA fragments (Jiang et al., 1999). The acquired sequences were compared with the published HP1a cDNA by BLAST2Seq (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify those <95% identical. The cDNA clones were then completely sequenced to search for corresponding gene(s) in the *M. sexta* genome (Cao et al., 2015). The signal peptide and domain structure were predicted by SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi). Based on the aligned catalytic domains, residues corresponding to Ser¹⁹⁰, Gly²¹⁶, and Gly²²⁶ of bovine chymotrypsin were identified in HP1a or HP1b to predict substrate specificity (Perona and Craik, 1995). The disulfide linkage pattern and proteolytic activation site of proHP1a or proHP1b were predicted based on alignment of HP1 and PAPI

sequences (Jiang et al., 1998). Molecular mass, isoelectric point, and other biochemical features of the predicted HP1's light and heavy chains were calculated using ProtParam (<http://web.expasy.org/protparam/>).

2.3. Quantitative real-time polymerase chain reaction (qPCR)

cDNA samples (each equivalent to starting with 50 ng total RNA) were incubated with 1× iTag Universal SYBR Green Supermix (Bio-Rad) and specific primers (0.5 μM each) in triplicate in each 10 μl reaction. The primers were: j055 (5'-CTCAGGCCGAGTCTTTGAGATACA) and j056 (5'-ACTTCATGGACTTGGCTCTCTGAC) for rpS3; j770 (5'-CATTGCACGAGAAGATGG) and j771 (5'-GTCGTGATGGTAGTTGGATA) for HP1a; j768 (5'-TCATTGCACTGACAAGTTT) and j769 (5'-TCGTGATGGTAGTTCAGTG) for HP1b. The thermal cycling conditions were: denaturation at 95°C for 30 s followed by 50 cycles of 95°C for 10 sec and 60°C for 30 sec. After the PCR was complete on a CFX Connect Real-Time PCR Detection System (Bio-Rad), melt curves of the products (156, 150, and 150 bp) in all reactions were examined to ensure proper shape and TM values. Absence of primer-dimer and correct sizes of the products were confirmed by agarose gel electrophoresis. Amplification efficiencies (E), measured by amplifying a cDNA sample diluted to 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ using the specific primer pairs under the same conditions, were 93.8% (rpS3), 103.5% (HP1a), and 100.1% (HP1b). Relative HP1 mRNA levels were calculated as: $(1 + E_{rpS3})^{Ct_{rpS3}} / (1 + E_{HP1})^{Ct_{HP1}}$ (Rieu and Powers, 2009).

2.4. Purification of *M. sexta* proHP1 from larval hemolymph

Hemolymph (40 ml) from cut prolegs of day 3, 5th instar naïve larvae (*c.a.* 50) was collected into 1.1–1.3 volume of ice-cold 100% saturated ammonium sulfate (AS). Precipitated proteins in the suspension was separated from soluble components by centrifugation at 12,000g for 20 min. The pellet was dissolved in 50 ml, 10 mM Tris-HCl (pH 8.0) containing 15% saturated AS. After centrifugation, AS saturation of the supernatant was slowly adjusted to 38%. The protein precipitate harvested by centrifugation was dissolved in 20 ml of HT buffer (pH 6.8, 5 mM potassium phosphate, 0.5 M NaCl), supplemented with 0.01% 1-phenyl-2-thiourea and 1 mM benzamidine, and dialyzed against the same buffer overnight at 4°C. After removing flocculent substances by centrifugation at 15,000g for 30 min, the supernatant was applied to a hydroxylapatite column (2.5 cm i.d.×10 cm, Bio-Rad) equilibrated in HT buffer. Following a washing step with 100 ml HT buffer, bound proteins were eluted at 0.4 ml/min for 6.7 h with a linear gradient of 5–100 mM potassium phosphate, pH 6.8, 0.5 M NaCl (160 ml). After 10% SDS-PAGE and immunoblot analysis, the proHP1 fractions (3 ml/tube) were pooled and precipitated by adding solid AS to a final saturation of 50%. The precipitate, collected by centrifugation at 15,000g for 30 min, was dissolved in 3 ml of 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl and separated by gel filtration chromatography on a Sephacryl S100-HR column (2.5 cm i.d.×100 cm) equilibrated with the same buffer. The proHP1 fractions (3 ml/tube) were combined and dialyzed against 20 mM ammonium formate, pH 7.8, 0.005% Tween-20 and applied to a Mono Q 5/50 GL column equilibrated with the same buffer. After washing with 10 ml of the buffer, a linear gradient of 20–360 mM ammonium formate, pH 7.8, 0.005% Tween-20 (48 ml) was

employed to elute proteins. The proHP1 fractions (1.2 ml each) were lyophilized, dissolved in 0.1 ml 20 mM Tris-HCl (pH 7.5), and stored at -80°C .

2.5. Characterization of purified *M. sexta* proHP1

The purified natural (n) proHP1 was subjected to 10% SDS-PAGE, followed by Coomassie Blue staining or immunoblotting using 1:3000 diluted HP1 antiserum (Jiang et al., 1999), respectively. MALDI-TOF mass spectrometry was performed at OSU Recombinant DNA/Protein Resource Facility. Individual proHP1 bands were excised from the stained reducing gel, treated with 100 mM iodoacetamide, and digested with 100 ng sequencing-grade trypsin in 25 mM ammonium bicarbonate at 37°C for 16 h. After desalting, the peptides were analyzed on a Voyager Elite mass spectrometer (Perkin-Elmer). Mass data were acquired in reflector mode within the range of 500 – 3,000 Da. Molecular masses of the peptides from proHP1 were calculated (<http://prospector.ucsf.edu/>) and compared with those of observed monoisotopic peaks to locate their positions. To detect possible *N*-linked glycosylation, proHP1 (0.1 μg) was treated with 1 $\times$ glycoprotein denaturing buffer (Sigma- Aldrich) at 100°C for 10 min. After adding 1/10 volume each of 10 $\times$ G7 buffer (50 mM sodium phosphate, pH 7.5) and 10% Nonidet P-40, the protein was incubated with 2.5 μl PNGase F (Sigma-Aldrich) at 37°C for 1 h. To detect *O*-linked glycosylation, proHP1 (0.1 μg) was reacted with 2 μl *O*-glycosidase (Sigma) in 1 $\times$ reaction buffer at 37°C for 3 h. The reaction mixtures, as well as untreated control, were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane, and detected with HP1a antibodies.

2.6. Production of *M. sexta* proHP1a/b and their catalytic domains (catHP1a/b) in the baculovirus expression system

ProHP1a and proHP1b were amplified from their respective full-length cDNAs by PCR using forward (j1351 5'-GAATTCGAGATTACTCCGAACTGCA-3', *Eco*RI site underlined) and reverse (j587, 5'-CTCGAGCCGTGCATTCTCCATG-3', *Xho*I site underlined) primers. The catalytic domains (catHP1a and catHP1b) were amplified using forward (j586, 5'-CAATTGGCGGCCCGCCAGCAA-3', *Mfe*I site underlined) and reverse (j587) primers. The amplified fragments were subcloned into pGEM-T vector (Promega) and, after sequence validation, retrieved as *Eco*RI-*Xho*I and *Mfe*I-*Xho*I fragments for directional insertion to the *Eco*RI (compatible with *Mfe*I) and *Xho*I sites in pMFH6 (Lu et al., 2008). The recombinant plasmids (proHP1a/pMFH6, proHP1b/pMFH6, catHP1a/pMFH6, and catHP1b/pMFH6) were used to generate bacmids and high-titer baculoviruses. ProHP1a/b and catHP1a/b were separately purified from 500 ml of the cell culture media by cation exchange and Ni^{2+} affinity chromatography (Sumathipala and Jiang, 2010) and concentrated using Amicon ultracentrifugal 30K and 10K MWCO filter devices (Millipore). Following buffer exchange to 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, the proteins were stored at -80°C in aliquots.

2.7. Elicitor-independent proPO activation by natural (n-) proHP1, proHP1a/b or catHP1a/b

Aliquots of cell-free hemolymph from day 2, fifth instar naïve larvae or day 3, fifth instar induced larvae (*Section 2.1*) were incubated with purified n-proHP1 (400 ng) in 0.001% Tween- 20, 1 mM CaCl_2 , 20 mM Tris-HCl, pH 7.5, for 1 h at room temperature. PO activity was determined using dopamine as a substrate on a 96-well microplate reader (Jiang et al.,

2003a). Similarly, naïve larval plasma was incubated with proHP1a/b (100 ng) or catHP1a/b (50 ng) for 30 min. To detect the difference in cetylpyridinium chloride (CPC) induced PO activity from naïve larval plasma and the same amount of purified proPO, the samples were separately mixed with the 150 µl of dopamine solution with or without the detergent for PO activity measurement.

2.8. Measurement of CPC-induced amidase activities

The purified proHP1a/b or catHP1a/b was incubated with 0.2% CPC (1 µl) and buffer for 5 min at room temperature. The amidase activity was measured using a synthetic substrate *N*-acetyl-Leu-Asn-Leu-His-*p*-nitroanilide (LDLH p NA, a kind gift from Dr. Kanost at Kansas State University) by monitoring A₄₀₅ in the kinetic mode on a plate-reader. One unit activity is defined as A₄₀₅/min = 0.001.

2.9. CPC/CTAB-induced cleavage of proHP1a/b and proHP6

The purified proHP1a/b and/or proHP6 (Wang and Jiang, 2008) were incubated with 6 mM CPC or CTAB (*i.e.* cetyltrimethyl ammonium bromide) and 20 mM Tris-HCl, pH 7.5, 20 mM NaCl at 37°C for 4 h. Each reaction was treated with SDS sample buffer with or without DTT and subjected to immunoblot analysis using diluted HP6 antiserum as the primary antibody. In a separate reaction, the 29 kDa cleavage product of proHP6 was generated, separated by SDS-PAGE from the other proteins, transferred onto a PVDF membrane, and sequenced by automated Edman degradation at Nevada Proteomics Center.

2.10. Proteolytic activation of proHP6, proPAP1 and proHP8 induced by catHP1a/b or *M. luteus* in larval hemolymph

The recombinant proPAP1, proHP6 and proHP8 were prepared according to Wang and Jiang (2001 and 2008). Larval hemolymph, buffer, proHP6, proPAP1, and/or proHP8 were incubated with catHP1a/b (50 ng) or *M. luteus* (1 µg) for 30 min at room temperature. The reaction mixtures and controls were subjected to 10% SDS-PAGE (+/- DTT) and immunoblot analysis using diluted antiserum against HP6, PAP1 or HP8 as the primary antibody.

3. Results and discussion

The initial excitement brought by the cDNA cloning of *M. sexta* HP1a (and HP2) in 1993 faded away quickly after subsequent experiments yielded negative results. HP1a antibodies did not detect proteolysis of proHP1 in the larval plasma after proPO activation was elicited. Lacking an affinity tag in the original Bac-to-Bac system, recombinant proHP1a from the baculovirus-infected insect cells was purified to less than 10% of total proteins, a purity too low for reliable functional tests. Partial purification of proHP1 from plasma led to the finding of a cleaved proHP1 twice, but its two chains were detached even in the absence of a reducing agent (Fig. S1). This atypical cleavage product, yielded during purification, did not cut purified proPO or form an SDS-stable complex with *M. sexta* serpin-1J, one of the 12 splicing variants that blocked proPO activation (Jiang and Kanost, 1997). However, these negative results did not exclude the possibility that the typical HP1 (*i.e.* putatively active HP1 cleaved between R¹⁵⁴ and V¹⁵⁵ with an interchain disulfide bond linking the N-

terminal light and C-terminal heavy chains) or proHP1* (* denotes the zymogen in an active conformation) plays a role in the proPO activation system (see below).

3.1. Cloning and biochemical properties of *M. sexta* HP1b

Intrigued by the minor differences in HP1 cDNA fragments (Jiang et al., 1999), we analyzed the 33 HP1 clones (Jiang et al., 2005) and found 31 were 98–100% identical to the published HP1a cDNA (data not shown) and the other two were about 90% identical. While allelic variations may account for the 6-nucleotide difference (4 synonymous) between #371 and #681, the 75-nucleotide (or 6.4%) difference between the coding regions of #681 and HP1a actually comes from two separate genes (Cao et al., 2015). The HP1b cDNA sequence (#681) has an open reading frame of 1,167 bp long, encoding a 388-residue protein identical in size to HP1a (Fig. 1). Following a 14-residue signal peptide, the mature proHP1b has a calculated molecular mass of 41,844 Da and isoelectric point of 5.38, lower than those of proHP1a (41,934 Da, 5.67). Both proteins are predicted to be glycosylated at Thr¹⁰⁹, Thr¹¹⁰, Thr¹²⁴, Thr¹³³, Thr¹⁴⁸, Thr¹⁴⁹, Asn²¹⁹, and Asn²⁷⁵. Based on the sequence alignment, proHP1a and proHP1b are predicted to be activated by a trypsin-like SP that cuts between Arg¹⁵⁴ and Val¹⁵⁵ to produce a 140-residue light chain (15,312 Da for HP1a; 15,311 Da for HP1b) and a 234-residue heavy chain (26,640 Da for HP1a; 26,551 Da for HP1a), linked by an interchain disulfide bridge. Their N-terminal light chain contains a group-1b clip domain, together with the respective C-terminal catalytic domain, making each protein a member of the CLIPD subfamily (Cao et al., 2015). HP1a and HP1b may cut after a positively charged residue (Arg or Lys) due to its negatively charged S1 pocket (Asp³³², Gly³⁶⁰ and Gly³⁷⁰) (Fig. 1). HP1a is 89.7% identical and 95.1% similar to HP1b in amino acid sequence.

3.2. Expression profiles of HP1a and HP1 in tissue and plasma samples

We previously detected HP1a and HP1b transcripts in control and induced fat body by RNA-Seq analysis and the read numbers were 1/50–300 those of the hemocyte samples, probably reflecting hemocytes trapped in fat body tissues (Zhang et al., 2011). The ratios of HP1a and HP1b read numbers were 6.94 and 1.31 in hemocytes from the naïve and induced larvae, respectively. There was a 57% decrease in HP1a mRNA level in hemocytes and 17% decrease ($p < 0.05$) in HP1a protein level in plasma after the immune challenge (He et al., 2016). In contrast, there was a 2.3-fold increase in HP1b mRNA and 17% increase ($p > 0.05$) in HP1b protein. The down-regulation of HP1a expression ($p < 0.05$) and HP1b up-regulation ($p > 0.05$) were also detected by qPCR analysis (Fig. 2A). Tissue specific expression of HP1a and HP1b was considerable in day 3, 5th instar larvae. Their mRNA levels in integument, fat body, midgut, Malpighian tubules, muscle, and nerve tissues were 0–5% of those in hemocytes (Fig. 2B). The HP1a mRNA in trachea was slightly higher, accounting for 8% of the levels in hemocytes. These data are mostly consistent with the expression profiles of HP1a and HP1b (Cao et al., 2015; Fig. S2). One potential discrepancy was the detection of both transcripts in muscle of day 0.5, 5th instar larvae. Since their mRNA levels (*i.e.* FPKM) are estimated to be 1/3 of those in hemocytes, we do not believe this is an artifact of hemocyte contamination. Neither is it clear why muscles of the young 5th instar express the two genes. HP1a transcript levels in hemocytes of the 4th and early 5th instar feeding larvae were significantly higher than those of HP1b (Fig. 2C). There was an HP1b mRNA peak in the end of feeding stage and beginning of wandering stage, as a result

of possible hormonal regulation. Such infection-independent expression at this stage is common for some immune inducible genes (Zhang et al., 2015; Rao et al., 2015; Cao et al., 2015; He et al., 2015). Interestingly, expression of HP1b, rather than HP1a, was induced upon immune challenge (Fig. 2A). Their hemocyte expression in the late wandering and entire pupal stages was shut down completely (Fig. 2C). Immunoblot analysis revealed a notable increase in proHP1 abundance in cell-free hemolymph between 4th and early 5th instar but no substantial increase was observed between early 5th instar and prepupal stages (Fig. 2D). These data agreed well with the developmental regulation in hemocytes. While the cross-reactivity of HP1a antibodies to HP1b made it impossible to distinguish the two proenzymes, quantitative proteomic analysis by nanoLC-MS/MS (He et al., 2016) allowed us to estimate that proHP1a was 8.8- and 6.3-fold higher in abundance than proHP1b in the naïve and induced day 3, 5th instar larvae, respectively.

3.3. Purification and characterization of proHP1

In order to explore the functions of HP1a and HP1b, we developed a protocol to isolate proHP1 from cell-free hemolymph of day 3, naïve larvae (Table 1). Over 90% of plasma proteins was removed by 15–38% (NH₄)₂SO₄ fractionation in the first step. After dialysis, loading and washing, bound proHP1 was eluted as a single peak from the hydroxylapatite column in the first part of the 5–100 mM gradient of potassium phosphate (Fig. 3A). ProHP1 in the pooled sample was separated into two peaks on the gel filtration column. While the small one came off soon after the column's void volume, over 95% of the proHP1 eluted as a major peak in the later fractions (Fig. 3B). Therefore, a small portion of the proenzyme formed a high M_r complex, which dissociated in the reducing condition. This was consistent with the further formation of a covalently linked, proHP1-containing immune protein complex in plasma (He et al., 2016). Subsequent resolution of the major proHP1 peak by anion exchange chromatography on a Mono-Q column yielded an A_{280nm} peak of proHP1 (Fig. 3C). The purified protein was then used as a standard to determine proHP1 concentrations in samples taken at different stages of purification (Table 1, Fig. 3D). From 40 ml of larval hemolymph (1.2 g total protein, estimated proHP1 concentration: 27 µg/ml), we obtained 0.14 mg proHP1 at a yield of 13% and purification of 1,100-fold.

The purified proHP1 migrated as a 47–48 kDa doublet on 10% SDS-PAGE under the reducing condition. In the absence of DTT, the SDS-bound proenzyme adopted more compact structures with apparent M_r 's of 36 and 37 kDa (Fig. 3E). Equal intensity of the double bands did not support them being proHP1a and proHP1b correspondingly. As indicated above, their ratio was 8.8:1 in the hemolymph from naïve larvae, from which proHP1 was isolated. Removal of *N*-glycans in the precursor by PNGase F converted the doublet to a single band at 46 kDa (Fig. 3F), suggesting that Asn²¹⁹ and Asn²⁷⁵ were modified. This is consistent with the binding of proHP1 to Concanavalin A (data not shown), a lectin recognizing mannose in *N*-glycans. On the other hand, none of the predicted *O*-linked sites appeared to be glycosylated. Results of the MALDI-TOF mass fingerprint analysis (Fig. 1) and deglycosylation test were consistent. Both bands in the doublet contained proHP1a and no proHP1b-specific monoisotopic peak was observed in the spectra, suggesting that proHP1b level was below the detection limit and/or a few peptides unique to proHP1b did not ionize well. During purification, we did not find evidence that

proHP1b had been separated from proHP1a. In comparison with the major peak, amount of the high M_r complex was estimated to be less than 1/100 (certainly not 1/8.8). As reported above, both proteins had similar biochemical properties such as M_r , pI, sequence and folding, likely. Therefore, we use “n-proHP1” or simply “proHP1” to describe the proteins purified from hemolymph.

3.4. Activation of proHP1 in vitro and its possible involvement in melanization

We have tried trypsin, *M. sexta* PAP1 and PAP3 to activate the purified proHP1 by cutting between Arg¹⁵⁴ and Val¹⁵⁵. But none of the treatments generated active HP1 that hydrolyzed peptidyl-*p*-nitroanilide substrates with the scissile bond after Arg or Lys under various conditions (data not shown). The failures led us to postulate that the proHP1 activation either requires a highly specific endogenous protease not yet tested or adopts an unusual mechanism.

We supplemented hemolymph from larvae with two batches of the proHP1 purified from hemolymph and observed small or statistically insignificant PO activity increases (Fig. 4A). To test this whimsical increase, we expressed proHP1a and its catalytic domain (catHP1a) (Fig. 4B). We also produced recombinant proHP1b and catHP1b to examine if the inducible isoform plays a more active role in the immune system. Based on our design, the mature proenzymes should have the sequence of GIRDYS...NARLEHHHHHH, in which the underlined part is identical to the natural proteins. Restricted by the vector and added restriction site sequence, the catHP1a/b (GIGGRPA...NARLEHHHHHH) is not identical to the conserved starting sequences (*e.g.* IIGG) of SP catalytic domain (Herter et al., 2005; Ross et al., 2003), but is closely similar. In contrast to the small increases caused by the n-proHP1, adding any of the four proteins led to a significant PO activity increase in plasma from naïve larvae (Fig. 4C). The increases caused by the catalytic domains were approximately 3-fold higher than those caused by the corresponding zymogens. We suspected that the recombinant proHP1a/b had certain level of high M_r complexes and removing them from the monomeric proHP1 by gel filtration chromatography (Fig. 3B) could be responsible for the discrepancy. Analogously, the recombinant peptidoglycan recognition protein-1 and microbe binding protein of *M. sexta* gave rise to proPO activation in the absence of bacterial cell wall components (Sumathipala and Jiang, 2010; Wang et al., 2011). The endogenous pattern recognition proteins and proHP1 in plasma apparently do not initiate the system under the normal physiological condition. Perhaps, spontaneous oligomerization of these system components can trigger melanization and additional association of the oligomers on microbial surfaces enhance the potency and locality of this response (Yu et al., 2003; Clark and Strand, 2013; He et al., 2016).

The possible involvement of *M. sexta* proHP1 in melanization is supported by an independent study (Suwanchaichinda et al., 2010). After *M. sexta* plasma sample was added to the conditioned medium of *Drosophila* S2 cells expressing proHP1a, elicitor-independent melanization occurred and the PO activity became significant higher than the control of S2 cells only. In another negative control, after the proHP1a expression had been blocked by its double stranded RNA, melanization did not occur.

3.5. Exploration of proHP1 activation mechanism

One of our general observations regarding proPO activation is that a larval plasma sample, after being treated by cetylpyridinium chloride (CPC, a cationic detergent), generates a much higher level of PO activity than the same amount of purified proPO does (Fig. 5A). While the compound induces the active conformation of proPO without cutting it (Hall et al., 1995), other plasma factors in the system may also be activated by the detergent to account for the enhancement. Therefore, we tested whether or not CPC treatment can induce proHP1 activation and found, interestingly, proHP1a was auto-cleaved after incubation (Fig. 5B). (Due to its chemical nature, CPC is unlikely to cut proHP1 at a specific site by itself.) An amidase activity was detected at a significantly higher level in the CPC-treated proHP1a than proHP1a alone, which hydrolyzed Leu-Asp-Leu-His-*p*-nitroanilide (Fig. 5C). The induction was less pronounced between proHP1b and CPC-treated proHP1b. This is somewhat in agreement with the prediction that HP1a or HP1b has a trypsin-like specificity (Section 3.1): at pH 7.0, the ratio of neutral and positively charged His side chain ($pK = 6.0$) was 10:1 and the actual pK of the side chain in the primary substrate-binding pocket is unknown. Remarkably, LDLH p NA was synthesized according to the sequence preceding proHP6's proteolytic activation site, suggesting that proHP1a/b (or its auto-cleavage product) is an activating enzyme of proHP6 in *M. sexta*.

Incubation proHP1a with proHP6 in the presence of CPC yielded one immunoreactive band at 29 Da positions (Fig. 5B), similar in size to the heavy chain of HP6 (An et al., 2009). However, five cycles of automated Edman degradation of this band revealed the original N-terminus of mature recombinant proHP6 as Glu (3.45) - Asn (2.14) - Val (2.90) - Gly (1.16) - Asp (1.44), where the numbers in parentheses represent amounts of the major phenylthiohydantoin derivatives in picomoles detected in these cycles. In other words, proHP6 was cut by CPC-treated proHP1a at the first 2/3 of the protein, instead of 1/3 that exposes the HP6 catalytic domain starting with Ile-Leu-Gly-Gly. Incubation of proHP6 with CPC did not induce auto-processing (Fig. 5B). As a result of the incorrect processing by CPC-treated proHP1a, the two fragments from proHP6 (one invisible to the antibodies) stayed separated even in the absence of DTT. The regulatory and catalytic domains of correctly processed HP6 are tethered by an interchain disulfide bond (An et al., 2009). Conceivably, CPC must have altered one or both proenzymes' conformation incorrectly, so that proHP1 in the induced state was able to hydrolyze the synthetic substrate (LDLH p NA) but not the natural substrate proHP6 next to LDLH. We tested several other detergents (SDS, Triton-X114, Tween-20, NP40) and found only cetyltrimethyl ammonium bromide (CTAB, another cationic detergent) caused proHP6 cleavage by proHP1a at the incorrect site (Fig. 5D). The same results were obtained using proHP1b (data not shown).

3.6. Can the catalytic domain of proHP1a or proHP1b cleave proHP6 at the correct site?

Like their precursors, the catalytic domains were barely active as hydrolases, but the LDLH p NA hydrolysis increased considerably after CPC was added (Fig. 5C). Thus, overall folding of the domains appeared to be correct and induction of the catalytic conformation did not require the amino-terminal clip domain or interdomain linker.

Since the CPC/CTAB treatment must have distorted the delicate interaction between active proHP1a/b and its putative protein substrate, we further tested if catHP1a/b can cut proHP6. Unfortunately, the C-terminal domain of HP1a or HP1b did not cut proHP6, proHP8, or proPAP1 *in vitro* in the absence of CPC (Fig. S3 and data not shown). Since both domains induced about 3-fold higher PO activity than their zymogens did in the plasma from naïve larvae (Fig. 4C), we examined the reaction mixtures and found that proHP6 was somehow converted to the two-chain form (Fig. 6A) and, since the two chains migrated to a position close to proHP6's under non-reducing condition, the cleavage site appeared to be correct (Section 3.5). Furthermore, the catalytic domain of HP1b more efficiently converted proHP6 to HP6. As substrates of HP6 (An et al., 2009), proPAP1 and proHP8 were in turn activated (Fig. 4D). Active PAP1 then stimulated a positive feedback loop that generates active POs (Wang and Jiang, 2008). On the other hand, active HP8 converted pro-Spätzle to Spätzle that binds its receptor to activate the Toll signaling pathway (An et al., 2010).

It is unclear how catHP1a/b may interact with other plasma factors to activate proHP6. Like the intact proteins, the catalytic domains of HP1a and HP1b cut purified proHP6 at the wrong site in the presence of CPC (data not shown). The difference between proHP1a/b and catHP1a/b in causing plasma melanization (Fig. 4C) suggested that the proenzyme's amino-terminal region may attenuate the system activation caused by the catalytic domains alone.

3.7. A proposed model for proHP1's role in the proPO activation system

In summary, *M. sexta* HP1a and HP1b gene expression is differentially regulated in a tissue-specific and development-dependent manner. Upon immune challenge, the expression of HP1a and HP1b in hemocytes is suppressed and induced at the mRNA levels, respectively, consistent with 17% decrease and 17% increase in their protein levels. Although total amount of the two proteins decreased, proHP6 activation by proHP1a* and proHP1b* may increase since the latter is more efficient in converting proHP6 to HP6. Here we propose that a physiological or pathological cue leads to a conformational change from inactive (proHP1a/b) to active (proHP1a*/b*) without proteolytic cleavage (Fig. 7). The change and interaction with proHP6 may be similar but not identical to what is caused by CPC or CTAB, otherwise the detergent-treated proenzyme would have cut purified proHP6 at the right site. The amino-terminal clip domain and linker region may play a role in the molecular recognition or preventing excessive activation. Docking and oligomerization of the proenzymes on microbial surfaces may facilitate their structural transition in the cell-free hemolymph. The active proHP1 then cleaves *M. sexta* proHP6 at the correct site to generate HP6, which sequentially activates other members of the system including HP8, Spätzle, PAP1, and PO. Nature for the conformational transition in proHP1a/b is unclear at this time and needs to be explored in the future. Nonetheless, the most important achievement of this study is the establishment of proHP1a/b as a component of the serine protease system for proPO and Toll pathway activation in *M. sexta*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health Grants GM58634 and AI112662. We would like to thank Dr. Michael Kanost at Kansas State University for providing critical comments on the manuscript and the synthetic substrate of LDLH ρ Na for the activity assay. This article was approved for publication by the Director of the Oklahoma Agricultural Experiment Station and supported in part under project OKLO2450.

Abbreviations

AMP	antimicrobial peptide
AS	ammonium sulfate
CH, IH, CF and IF	control (C) and induced (I) hemocytes (H) and fat body (F)
NP and IP	cell-free hemolymph from naïve or induced larvae
CPC	cetylpyridinium chloride
CTAB	cetyltrimethylammonium bromide
DTT	dithiothreitol
FPKM	fragments per kilobase of exon per million fragments mapped
HP	hemolymph (serine) protease
LDLHρNa	<i>N</i> -acetyl-Leu-Asn-Leu-His- <i>p</i> -nitroanilide
PO and proPO	phenoloxidase and its precursor
PAP	proPO activating protease
qPCR	quantitative real-time polymerase chain reaction
rpS3	ribosomal protein S3
SP and SPH	serine protease and its noncatalytic homolog
SEC	serpin-enzyme complex

References

- An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR. Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. *J. Biol. Chem.* 2009; 284:19716–19726. [PubMed: 19487692]
- An C, Jiang H, Kanost MR. Proteolytic activation and function of the cytokine Spätzle in the innate immune response of a lepidopteran insect, *Manduca sexta*. *FEBS J.* 2010; 277:148–162. [PubMed: 19968713]
- Cao X, He Y, Hu Y, Zhang X, Wang Y, Zou Z, Chen Y, Blissard GW, Kanost MR, Jiang H. Sequence conservation, phylogenetic relationships, and expression profiles of nondigestive serine proteases and serine protease homologs in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 2015; 62:51–63. [PubMed: 25530503]

- Clark KD, Strand MR. Hemolymph melanization in the silkworm *Bombyx mori* involves formation of a high molecular mass complex that metabolizes tyrosine. *J. Biol. Chem.* 2013; 288:14476–14487. [PubMed: 23553628]
- Dunn P, Drake D. Fate of bacteria injected into naïve and immunized larvae of the tobacco hornworm, *Manduca sexta*. *J. Invert. Pathol.* 1983; 41:77–85.
- Gettins PG. Serpin structure, mechanism, and function. *Chem Rev.* 2002; 102:4751–4804. [PubMed: 12475206]
- Hall M, Scott T, Sugumaran M, Söderhäll K, Law JH. Proenzyme of *Manduca sexta* phenol oxidase: purification, activation, substrate specificity of the active enzyme, and molecular cloning. *Proc. Natl. Acad. Sci. USA.* 1995; 92:7764–7768. [PubMed: 7644492]
- He Y, Cao X, Li K, Hu Y, Chen YR, Blissard G, Kanost MR, Jiang H. A genome-wide analysis of antimicrobial effector genes and their transcription patterns in *Manduca sexta*. *Insect Biochem. Mol. Biol.* 2015; 62:23–37. [PubMed: 25662101]
- He Y, Cao X, Zhang S, Rogers J, Hartson S, Jiang H. Changes in the plasma proteome of *Manduca sexta* larvae in relation to the transcriptome variations after an immune challenge: evidence for high molecular weight immune complex formation. *Mol. Cell. Proteomics.* 2016 In press.
- Herter S, Piper DE, Aaron W, Gabriele T, Cutler G, Cao P, Bhatt AS, Choe Y, Craik CS, Walker N, Meininger D, Hoey T, Austin RJ. Hepatocyte growth factor is a preferred in vitro substrate for human hepsin, a membrane-anchored serine protease implicated in prostate and ovarian cancers. *Biochem. J.* 2005; 390:125–136. [PubMed: 15839837]
- Iwanaga S, Kawabata S, Muta T. New types of clotting factors and defense molecules found in horseshoe crab hemolymph: their structures and functions. *J. Biochem.* 1998; 123:1–15. [PubMed: 9504402]
- Jiang H, Kanost MR. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. *J. Biol. Chem.* 1997; 272:1082–1087. 1997. [PubMed: 8995406]
- Jiang, H.; Vilcinskas, A.; Kanost, MR. Immunity in lepidopteran insects. In: Söderhäll, K., editor. *Invertebrate Immunity*. Vol. 708. 2010. p. 181–204. *Adv. Exp. Med. Biol.*
- Jiang H, Wang Y, Kanost MR. Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: a bacteria-inducible protein similar to *Drosophila easter*. *Proc. Natl. Acad. Sci. USA.* 1998; 95:12220–12225. [PubMed: 9770467]
- Jiang H, Wang Y, Gu Y, Guo X, Zou Z, Scholz F, Trenczek TE, Kanost MR. Molecular identification of a bevy of serine proteinases in *Manduca sexta* hemolymph. *Insect Biochem. Mol. Biol.* 2005; 35:931–943.
- Jiang H, Wang Y, Kanost MR. Four serine proteinases expressed in *Manduca sexta* hemocytes. *Insect Mol. Biol.* 1999; 8:39–53. [PubMed: 9927173]
- Jiang H, Wang Y, Yu X-Q, Kanost MR. Prophenoloxidase-activating proteinase-2 (PAP-2) from hemolymph of *Manduca sexta*: a bacteria-inducible serine proteinase containing two clip domains. *J. Biol. Chem.* 2003a; 278:3552–3561. [PubMed: 12456683]
- Jiang H, Wang Y, Yu X-Q, Zhu Y, Kanost MR. Prophenoloxidase-activating proteinase-3 (PAP-3) from *Manduca sexta* hemolymph: a clip-domain serine proteinase regulated by serpin-1J and serine proteinase homologs. *Insect Biochem. Mol. Biol.* 2003b; 33:1049–1060. [PubMed: 14505699]
- Kanost MR, Jiang H. Clip-domain serine proteases as immune factors in insect hemolymph. *Curr. Opin. Insect Sci.* 2015; 11:47–55. [PubMed: 26688791]
- Kurata S. Fly immunity: recognition of pathogens and induction of immune responses. *Adv. Exp. Med. Biol.* 2010; 708:205–217. [PubMed: 21528700]
- Kwon TH, Kim MS, Choi HW, Joo CH, Cho MY, Lee BL. A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae. *Eur. J. Biochem.* 2000; 267:6188–6196. [PubMed: 11012672]
- Lu Z, Jiang H. Expression of *Manduca sexta* serine proteinase homolog precursors in insect cells and their proteolytic activation. *Insect Biochem. Mol. Biol.* 2008; 38:89–98. [PubMed: 18070668]
- O'Brien, D.; McVey, J. Blood coagulation, inflammation, and defense. In: Sim, E., editor. *The Natural Immune System, Humoral Factors*. New York: IRL Press; 1993. p. 257–280.

- Park, JW.; Kim, CH.; Rui, J.; Park, KH.; Ryu, KH.; Chai, JH.; Hwang, HO.; Kurokawa, K.; Ha, NC.; Söderhäll, I.; Söderhäll, K.; Lee, BL. Beetle immunity. In: Söderhäll, K., editor. *Invertebrate Immunity*. Vol. 708. 2010. p. 163-180. *Adv. Exp. Med. Biol*
- Perona JJ, Craik CS. Structural basis of substrate specificity in the serine proteases. *Protein Sci*. 1995; 4:337–360. [PubMed: 7795518]
- Rao XJ, Cao X, He Y, Hu Y, Zhang X, Chen YR, Blissard G, Kanost MR, Yu X-Q, Jiang H. Structural features, evolutionary relationships, and transcriptional regulation of C-type lectin-domain proteins in *Manduca sexta*. *Insect Biochem. Mol. Biol*. 2015; 62:75–85. [PubMed: 25554596]
- Rieu I, Powers SJ. Real-time quantitative RT-PCR: design, calculations, and statistics. *Plant Cell*. 2009; 21:1031–1033. [PubMed: 19395682]
- Ross J, Jiang H, Kanost MR, Wang Y. Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationship. *Gene*. 2003; 304:117–131. [PubMed: 12568721]
- Sumathipala N, Jiang H. Involvement of *Manduca sexta* peptidoglycan recognition protein-1 in the recognition of bacteria and activation of prophenoloxidase system. *Insect Biochem. Mol. Biol*. 2010; 40:487–495. [PubMed: 20416376]
- Suwanchaichinda C, Kelo L, Kanost MR. Possible involvement of *Manduca sexta* hemolymph protease-1 in melanization. *FASEB J*. 2010; 24:688.3.
- Takahashi D, Dai H, Hiromasa Y, Krishnamoorthi R, Kanost MR. Self-association of an insect β -1,3-glucan recognition protein upon binding laminarin stimulates prophenoloxidase activation as an innate immune response. *J. Biol. Chem*. 2014; 289:28399–28410. [PubMed: 25147183]
- Yu X-Q, Jiang H, Wang Y, Kanost MR. Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol*. 2003; 33:197–208. [PubMed: 12535678]
- Wang Y, Jiang H, Kanost MR. Expression and purification of *Manduca sexta* prophenoloxidase-activating proteinase precursor (proPAP) from baculovirus-infected insect cells. *Protein Expr. Purif*. 2001; 23:328–337. [PubMed: 11676609]
- Wang Y, Jiang H. A positive feedback mechanism in the *Manduca sexta* prophenoloxidase activation system. *Insect Biochem. Mol. Biol*. 2008; 38:763–769. [PubMed: 18625399]
- Wang Y, Lu Z, Jiang H. *Manduca sexta* prophenoloxidase activating proteinase-3 (PAP3) stimulates melanization by activating proPAP3, proSPHs, and proPOs. *Insect Biochem. Mol. Biol*. 2014; 50:82–91. [PubMed: 24768974]
- Wang Y, Sumathipala N, Rayaprolu S, Jiang H. Recognition of microbial molecular patterns and stimulation of prophenoloxidase activation by a β -1,3-glucanase-related protein in *Manduca sexta* larval plasma. *Insect Biochem. Mol. Biol*. 2011; 41:322–331. [PubMed: 21296155]
- Wang Y, Zou Z, Jiang H. An expansion of the dual clip-domain serine proteinase family in *Manduca sexta*: gene organization, expression, and evolution of prophenoloxidase activating proteinase-2, hemolymph proteinase 12, and other related proteinases. *Genomics*. 2006; 87:399–409. [PubMed: 16324822]
- Zhang S, Cao X, He Y, Hartson S, Jiang H. Semi-quantitative analysis of changes in the plasma peptidome of *Manduca sexta* larvae and their correlation with the transcriptome variations upon immune challenge. *Insect Biochem. Mol. Biol*. 2014; 47:46–54. [PubMed: 24565606]
- Zhang S, Zhang X, Gunaratna R, Najjar F, Wang Y, Roe B, Jiang H. Pyrosequencing-based expression profiling and identification of differentially regulated genes from *Manduca sexta* a lepidopteran model insect that lacks genome sequence. *Insect Biochem. Mol. Biol*. 2011; 41:733–746. [PubMed: 21641996]
- Zhang X, He Y, Cao X, Gunaratna RT, Chen YR, Blissard G, Kanost MR, Jiang H. Phylogenetic analysis and expression profiling of the pattern recognition receptors: Insights into molecular recognition of invading pathogens in *Manduca sexta*. *Insect Biochem. Mol. Biol*. 2015; 62:38–50. [PubMed: 25701384]

Highlights

- Identification of *M. sexta* HP1b, an isoform of HP1a 90% identical in protein sequence;
- Hemocyte-specific, development/immunity-dependent expression of the two genes;
- Functional characterization of proHP1a/b as a component of the proPO activation system;
- Proteolytic activation of proHP6 possibly by proHP1a/b in an active conformation;

```

1      MIFVFLLLLVSVCAEKGDYSELQHPAWEDIIEEGMHIGGGRSKRFIQLNPNQGNVAYQA
      -----T-----L-----
      a          b          c          b          ac      ++
61     CTLPSGKQGHCRHLRFCIQEDFKQDFVKFMNYVCVIQGGSMGVCCPEDTTVGGPEGLAGD
      ---N-----D-----I-----R---A---D-----E
          +          +          #      ++
121    LPATAPQEGEDETLLKINQAQNRCGLSTRAQGR | VFGSRPANPREWPWMASITPEGFEQ
      -----I--S-----P---|---D-----Y-R
          d          d          +
180    YCGGVLIITDRHVLTAAHCTRRWEANELYVRLGEYDFKRTNDTRSYNFRVVEKVQHVDFEI
      -----E-----=-DKF-----F---T
                              #          +
240    SNYHHDIAILKLDKPAIFNTYVWPICLPPPGLSIENETVTVIGWGTQWYGGPHSHVLMEV
      L-----=-----T-----SF---D--I-L--
                              e          e          @ f
300    SFPIWTHQNCIEVHTNSIFDESICAGGHEGGRDACQGDSGGPLMYQMPSGRWAVVGIVSW

```

Fig. 1. Comparison of *M. sexta* HP1a and HP1b sequences

Amino acid residues of HP1a (*upper*) are shown in single letter abbreviations in the alignment, with the identical ones in HP1b (*lower*) marked “-”. The secretion signal peptide is underlined. The six absolutely conserved Cys residues in the clip domain are sequentially labeled a, b, c, b, a, and c to indicate possible formation of the three disulfide bonds between C1–C5, C2–C4, and C3–C6. Similarly, the six Cys in the catalytic domain are labeled d, d, e, e, f, and f to indicate the conserved disulfide bond formation. The putative proteolytic activation site (AQGR¹³⁴ | V¹³⁵FGS in HP1a; PQGR¹³⁴ | V¹³⁵FDS in HP1b) is labeled with “|”. After predicted activation cleavage, the amino-terminal light chain and carboxyl-terminal heavy chain may be tethered by a conserved disulfide bridge (#–#) in the typical HP1a and HP1b (predicted). Putative *N*- and *O*-linked glycosylation sites are marked with “+”. The catalytic residues (**H**, **D**, and **S**) at the active site are double underlined, while residues determining the primary specificity are labeled with “’”. The stop codon is marked by an *asterisk*. The trypsinolytic fragments identified in the MALDI mass fingerprint analysis are indicated by wavy underlines.

Fig. 2. *M. sexta* HP1a and HP1b mRNA and protein levels in various samples from different life stages

(A) Immune inducibility. As described in *Sections 2.1 and 2.3*, total RNA samples of control and induced hemocytes (CH and IH) and fat body (CF and IF) were reverse transcribed to cDNA and analyzed by qPCR using HP1a- and HP1b-specific primers. Relative mRNA levels (indicated on *top*) were calculated based on C_t values from three biological replicates (3 larvae per sample) and plotted as a bar graph (mean $\pm$ SD, $n = 3$). HP1a, red; HP1b, gray (for A–C). (B) Tissue specificity. RNA samples of integument (I), fat body (F), hemocytes (H), midgut (Mg), Malpighian tubule (Mt), muscle (Mu), nerve tissue (N), salivary gland (S), and trachea (T) from day 3, fifth instar naïve larvae were prepared and analyzed by qPCR under the same conditions. (C) Developmental profiles. The HP1a and HP1b mRNA levels in hemocytes from larvae at different life stages were determined using the same method. “e”, early (5th feeding, day 1; 5th wandering, day 7; pupal, day 9); “m”, middle (4th instar, day 2; pupal, day 18); “l”, late (5th feeding, day 4; 5th wandering, day 9; pupal, day 27). For B and C, one biological sample with three technical replicates (mean $\pm$ SD, $n = 3$). (D) Protein abundance. ProHP1 in the plasma samples (1.0 μ l each, 2 samples per life stage) were subjected to 10% SDS-PAGE and immunoblot analysis using 1:2000 diluted HP1 antiserum as the first antibody. Sizes and positions of the molecular mass makers are indicated on the *left*.

Fig. 3. Purification and characterization of proHP1 from *M. sexta* larval plasma

(A) Separation of 15–38% $(NH_4)_2SO_4$ fraction of larval plasma on a hydroxylapatite column; (B) The pooled hydroxylapatite fractions resolved by gel filtration chromatography; (C) Separation of the combined Sephacryl S100 fractions on an HPLC Mono Q column. In A–C, fractions marked with red bars contained proHP1. (D) Coomassie blue staining (left) and immunoblot analysis (right) of proHP1 samples at different purification stages, separated by 10% SDS-PAGE. Lane M, prestained protein M_r standards; lane 1, 15–38% $(NH_4)_2SO_4$ fraction of larval plasma; lane 2, pooled hydroxylapatite fractions; lane 3, pooled Sephacryl S100 fractions; lane 4, pooled Mono-Q fractions. (E) Apparent M_r . The purified proHP1 (0.2 μ g) was treated with SDS sample buffer with or without DTT, separated by 10% SDS-PAGE, and detected with HP1-specific antibodies. (F) Deglycosylation. The protein (0.4 μ g) was treated with buffer (lane 1), PNGase F (lane 2) or O-glycosidase (lane 3), separated by 7.5% SDS-PAGE under the reducing condition, and analyzed using HP1 antibodies. In D–F, sizes and positions of the M_r markers are indicated on the left.

Fig. 4. Activation of proPO in plasma from naïve larvae by purified *M. sexta* HP1a and HP1b proenzymes and catalytic domains

(A) Cell-free hemolymph from naïve (N) or induced (I) larvae (0.5 μ l) was incubated with purified natural proHP1 (0.4 μ g in 2 μ l) and 21.5 μ l buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM CaCl_2 , 0.001% Tween-20) at room temperature for 60 min prior to the PO activity assay. The PO activities (mean $\pm$ SD, $n = 3$) of the plasma alone and proenzyme only were used as negative controls for comparison with the mixture by Students' t-test (*, $p < 0.05$; n.s. or not significant, $p > 0.05$). (B) The purified proHP1a (lane 1, 1.2 μ g), proHP1b (lane 2, 0.2 μ g), catHP1a (lane 3, 0.5 μ g), and catHP1b (lane 4, 0.3 μ g) were separated by 10% SDS-PAGE and stained with Coomassie blue. (C) The recombinant proteins (100 ng for proHP1a/b, 50 ng for catHP1a/b in 1.0 μ l) were individually incubated with naïve larval plasma (1.0 μ l) and the buffer (8 μ l) for 30 min, assayed for PO activities, and analyzed for significant differences as described in panel A. (D) Immunodetection of proHP6, proHP8

and proPAP1 processing in naïve larval hemolymph (NH, 1.0 µl) after incubation with catHP1a (50 ng in 1.0 µl) and the buffer (8.0 µl) for 30 min. The control (NH only) and reaction mixture were separated by 10% SDS-PAGE followed by immunoblot analysis using 1:3000 diluted antiserum against HP6, HP8, or PAP1 as the primary antibody. The same results (not shown) were obtained using catHP1b and NH. ◀, proenzyme; ●, catalytic domain; ■, light chain containing a clip domain; *, nonspecific.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 5. Amidase activities of and proHP6 processing by purified *M. sexta* HP1a and HP1b proenzymes and catalytic domains induced by cetylpyridinium chloride (CPC)

(A) PO activities of purified proPO (60 ng) and naïve larval hemolymph (NH, 1 µl, containing *c.a.* 60 ng proPO) were measured in 150 µl of dopamine solution with or without 0.1% CPC. (B) The proHP1a (200 ng in 1 µl) and/or proHP6 (100 ng in 1 µl) were incubated with 0.2% CPC (1 µl) and buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 8 µl) were incubated at 37°C for 4 h, half of each reaction was treated with SDS sample buffer with DTT and the other half without DTT. Following 10% SDS-PAGE and electrotransfer, the blot was incubated with 1:3000 diluted HP6 antiserum (which crossreacted with proHP1 sometimes) as the primary antibody, 1:3000 diluted goat-anti-rabbit IgG conjugated to alkaline phosphatase, and NBT-BCIP substrate solution. ◀, proHP6; ■, proHP1; ○, N-terminal fragment of the atypical HP6 (*i.e.* HP6×); ●, small (antibody crossreactive) and large (antibody unrecognized) fragments of the atypical HP1 (HP6×, marked □), which were connected under nonreducing condition. (C) The purified proteins (0.5 µg for proHP1a/b, 0.3 µg for catHP1a/b in 2 µl) were separately incubated 1 µl of 0.2% CPC, buffer (10 µl, 20 mM Tris-HCl, pH 7.0, 20 mM NaCl) for 5 min at room temperature. The protein alone and CPC only controls were included in the experiment. Amidase activities (mean ± SD, n = 3) were determined by adding 150 µl, 100 µM LDLH-*p*-nitroanilide in the buffer for proHP1a and catHP1a. Single reactions were performed for proHP1b and catHP1b, due to limitation

of the purified proteins. **(D)** As described in panel B, proHP1a (100 ng in 1 μ l), proHP6 (200 ng in 1 μ l), buffer (7 μ l), and 1 μ l of 6 mM CPC, CTAB or SDS were incubated for immunoblot analysis. Like SDS, 1 μ l of 6 mM Triton-X114, Tween-20, or NP40 did not induce proHP1a to cleave proHP6 (data not shown). There was no difference between proHP1a and proHP1b in terms of cutting proHP6 at the wrong site in the presence of CPC or CTAB and the other four detergents did not cause the proHP6 cleavage (data not shown).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 6. Cleavage of *M. sexta* proHP6 in plasma by catHP1a and catHP1b
Induced larval hemolymph (IH, 1 μ l), proHP6 (0 or 1 μ l, 100 ng/ μ l), and buffer (20 mM Tris-HCl, pH 7.0, 20 mM NaCl, 8 or 7 μ l) were incubated with *M. luteus* (1 μ g in 1 μ l) or catHP1a/b (50 ng in 1 μ l) in 10 μ l reactions for 30 min at room temperature. IH alone was included in the experiment as a control. Half of each sample was treated with SDS sample buffer with DTT and the other half without DTT. Following 10% SDS-PAGE and electrotransfer, the membrane was subjected to immunoblotting using 1:3000 diluted HP6 antiserum as the primary antibody. $\blacktriangleleft$, proHP6 (Note: the natural is slightly smaller than the recombinant); $\bullet$, the HP6 catalytic domain. The regulatory light chain (antibody unrecognized) was connected with the catalytic heavy chain as active HP6 ($\blacksquare$) under nonreducing condition. Bands representing a putative serpin-enzyme complex (SEC) are indicated.

Fig. 7. A model for nonproteolytic proHP1 activation, HP6 generation, induced AMP synthesis, and PO-catalyzed melanization

Table 1

Purification of *M. sexta* proHP1a from larval hemolymph^{*}

sample	volume (ml)	protein (mg/ml)	proHP1 (μg/ml)	yield (%)	purification (fold)
15–38% (NH ₄) ₂ SO ₄ fraction	21.9	1.50	32.8	40.0	874
pooled hydroxylapatite	3.77	1.05	3.96	100.0	377
Sephaeryl S100	25.9	0.02	0.52	8.0	207
Mono-Q ^{**}	3.6	0.042	0.15	42.0	150
				17.2	37.5

^{*} : Most proteins in the plasma (*c.a.* 30 mg/ml, 40 ml) were removed by 15–38% (NH₄)₂SO₄ fractionation, whereas *c.a.* 80% of proHP1 was retained in this step.

^{**} : After lyophilization, the purified proHP1a was dissolved in 0.3 ml at a final concentration of 0.455 mg/ml.