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Regulation of phenoloxidase activity by high- and low-molecular-weight inhibitors from the larval hemolymph of *Manduca sexta*

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Abstract

Insect phenoloxidases (POs) generate quinones and other reactive intermediates to immobilize and kill invading pathogens and parasites. Due to the presumed cytotoxicity of these compounds, PO activity and its proteolytic activation have to be regulated as a local, transient reaction against nonself in order to minimize damage to the host tissues and cells. We identified a *Manduca sexta* cDNA encoding a polypeptide sequence with its carboxyl-terminal 33 residues similar to the housefly phenoloxidase inhibitor (POI). The recombinant POI, secreted into the *Escherichia coli* periplasmic space along with its fusion partner DsbC, was released by osmotic shock and isolated by nickel affinity chromatography. Following enterokinase digestion and protein separation, the POI was purified to near homogeneity in a soluble form which inhibited *M. sexta* PO at a high concentration. We then produced the inhibitor using a modified baculovirus-insect cell system and isolated the glycoprotein from the conditioned medium. Deglycosylation coupled with inhibition assay revealed that *O*-glycosylation only moderately increased its inhibitory activity. While this led us to speculate the role of Tyr⁶⁴ hydroxylation, we were unable to modify the recombinant protein with tyrosine hydroxylase or purify *M. sexta* POI (Tyr⁶⁴dopa) from the larval plasma. Instead, we isolated a low-*M_r*, heat-stable compound which strongly inhibited PO. The wavelength of maximum absorbance is 257 nm for the inhibitor. These data suggest that the down-regulation of PO activity in *M. sexta* is achieved by two mechanisms at least.

Keywords

Melanization; Insect immunity; Hemolymph protein; Quinone cytotoxicity

Introduction

Tyrosinase-type phenoloxidases (POs) from insects are copper-containing enzymes that hydroxylate monophenols to *o*-diphenols (monophenol, oxygen, oxidoreductase, EC1.14.18.1) and oxidize *o*-diphenols to quinones (*o*-diphenol, oxygen, oxidoreductase, EC1.10.3.1) (Nappi and Christensen, 2005). Reactive quinones generated by POs polymerize to form eumelanin. Quinones may also crosslink nucleophilic compounds during wound healing and parasite encapsulation (Sugumaran, 1998; Lourenco et al., 2005). A recent study in *Aedes aegypti* suggested an essential role of POs in chorion melanization and hardening (Kim et al., 2005). While sclerotized cuticles effectively block most pathogens (Ashida and Brey, 1995), this physiochemical barrier is penetrated by others. Under such circumstances, melanization is often initiated in the plasma to reduce damage caused by the intruders and to impede their development. POs may also play a role in hemolymph coagulation, a process closely associated with wound healing (Li et al., 2002). A more recent study demonstrated that *Drosophila* PO

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was unnecessary for the preliminary soft clot formation, but was responsible for the clot hardening through crosslinking and melanization (Bidla et al., 2005).

Due to possible cytotoxicity of quinones generated during melanization, proPO activation and PO activity have to be tightly and elaborately regulated (Cerenius and Söderhäll, 2004). POs are synthesized by hemocytes as inactive zymogens. Upon wounding or infection, proPOs are activated via limited proteolysis through a serine proteinase cascade. Serpins regulate proPO activation by specific inhibition of the cascade components (Kanost et al., 2004).

Phenoloxidase inhibitor (POI) was first isolated from the housefly *Musca domestica* (Tsukamoto et al., 1992). It contains 38 amino acid residues stabilized by three disulfide bonds. One of the two tyrosine residues, Tyr³², is hydroxylated to dopa that is crucial for its inhibitory activity (Daquinag et al., 1995). Sequence comparison indicated the disulfide linkage pattern of POI may be identical to that of Ω -conotoxins from snails and spiders (Daquinag et al., 1999). *Manduca sexta* cuticle contains a high- M_r POI (Sugumaran and Nellaiappan, 2000). The heat-labile glycoprotein may inhibit insect, plant and fungal POs by forming detergent-resistant complexes. In addition, *M. sexta* quinone isomerase may form inactive complexes with POs (Sugumaran et al., 2000). The inhibition is reciprocal: POs also block the isomerase activities. Here, we report the production of *M. sexta* POI in *Escherichia coli* and insect cells, and the isolation of a low- M_r compound with strong inhibitory activity against *M. sexta* PO and mushroom tyrosinase

2. Materials and methods

2.1. Insect rearing and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supplies, and larvae were reared on an artificial diet (Dunn and Drake, 1983). Hemolymph from the induced larvae was collected and stored according to Jiang et al. (2003).

2.2. Expression and purification of *M. sexta* POI in *E. coli*

M. sexta POI cDNA (GenBank accession number: BE015616), kindly provided by Dr. Robertson at University of Illinois Urbana-Champaign, was amplified using j240 (5'-AGAGCCCGGGCAATTTGATAAGG-3') and j241 (5'-TTGTCGACCTATCCGGAGC-3'). After *SrfI* and *SalI* digestion, the amplified cDNA fragment was inserted to the same sites in pET40b (Novagen). The resulting plasmid, designated as POI/pET40b, was sequenced by the dideoxynucleotide termination method to verify the insert and design.

E. coli BL21(DE3) harboring the expression construct was cultured in 2L Luria-Bertani medium containing 100 μ g/ml ampicillin and induced with 1mM IPTG when A₆₀₀ reached 0.7. After 4 h, the bacterial cells were harvested by centrifugation at 5000 $\times$ g for 10 min. The pellets were resuspended in 500 ml 20 mM Tris-HCl, 20% sucrose, 1mM EDTA, pH 8.0, and stirred at room temperature for 10 min. Following centrifugation at 8000 $\times$ g for 10 min, the cells were resuspended in 500 ml ice-cold 5mM MgSO₄ and stirred at 4°C for 10 min. The periplasmic fraction was separated from the debris by centrifugation at 10,000 $\times$ g for 20 min.

The recovered supernatant was applied to a 10ml Ni²⁺-NTA agarose column equilibrated in 20mM Tris- HCl, pH 8.0, at 1 ml/min. After washing, bound proteins were eluted with 30 ml of 250mM imidazole in the equilibration buffer. The elution fractions were pooled and dialyzed against 20mM Tris-HCl, 20mM NaCl, 2mM CaCl₂, pH 7.6 (2.0 L each time for 12 h, twice). Enterokinase (40 U) (Novagen) was added to the dialyzed sample for specific cleavage between POI and its fusion partner DsbC. Following incubation at room temperature for 12h with gentle agitation, the reaction mixture was loaded onto an Ni²⁺-NTA column to remove uncleaved fusion protein and DsbC. The flow-through fraction was collected and further separated by reverse phase HPLC on a C₁₈ column (Bio-Rad). The POI peak was collected and lyophilized.

2.3. Expression and purification of *M. sexta* POI from insect cells

The plasmid pFH6 (Ji et al., 2003) was further improved for efficient secretion of recombinant proteins using honeybee melittin signal peptide. Briefly, j551 (5'-GA TCCATGAAATTCTTAGTCAACGTTGCCCTTGTTTTATGGTCGTATACATTTCTTACATCTATGCCGG) and j552 (3'-GTACTTTAAGAA TCAGTTGCAACGGGAACAAAAATACCAGCATATGTAAAGAATGTAGATACGGCCTTAA) were phosphorylated, annealed and inserted to the *Bam*HI and *Eco*RI sites of pFH6 to generate pMFH6. Plasmid DNA from the resulting transformants was verified by sequence analysis to encode Met-Lys-Phe-Leu-Val-Asn-Val-Ala-Leu-Val-Phe-Met-Val-Val-Tyr-Ile-Ser-Tyr-Ile-Tyr-Ala*Gly-Ile, where * denotes the signal peptidase cleavage site. The POI cDNA was amplified by PCR using j274 (5'-A GAATTCTAAGGGACGGAGTTGA) and j275 (5'-T CTCGAGTCCGGAGCCAGACAC). After T/A cloning and sequence confirmation, the cDNA fragment was retrieved by *Eco*RI-*Xho*I digestion and inserted to the same sites in pMFH6. *In vivo* transposition of the expression cassette, selection of colonies carrying the recombinant bacmid and isolation of bacmid DNA were performed according to manufacturer's protocols (Invitrogen Life Technologies). The initial viral stock (V^0) was obtained by transfecting *Spodoptera frugiperda* Sf21 cells with a DNA-CellFECTIN mixture, and its titer was improved through serial infections. The V^6 viral stock, containing the highest level of baculovirus, was stored at -70°C for further experiments. Expression conditions were optimized as described previously (Ji et al., 2003)

Sf21 cells (800 ml, $2.0 \times 10^6/\text{ml}$) in ultimate insect serum-free medium (Invitrogen Life Technologies) were infected with the recombinant baculovirus at a multiplicity of infection of 5 and grown at 27°C for 96 h with gentle agitation (100 rpm). The conditioned cell culture medium was recovered after centrifugation at $5000 \times g$ for 10 min. Following pH adjustment to 7.4 with 1M Na_2HPO_4 , the medium was applied onto an Ni^{2+} -NTA agarose column (15 ml), equilibrated with 50mM phosphate buffer, 0.5M NaCl, 10mM imidazole, pH 7.4. After washing with 90 ml of the same buffer, bound proteins were eluted from the column with a linear gradient of 10–500mM imidazole in the phosphate buffer (300 ml). The fractions exhibiting PO inhibitory activity were combined and separated by reverse phase HPLC as described above. Protein peaks were collected manually for the inhibition assay. The POI fractions were combined, lyophilized and dissolved in 100 μl of 20mM Tris-HCl, pH 7.5.

2.4. Isolation of a low- M_r POI from *M. sexta* hemolymph

The hemolymph from day 3, fifth instar *M. sexta* larvae injected with a mixture of formaldehyde-killed *E. coli* (3×10^7 cells), *M. luteus* (30 μg), and curdlan (30 μg) in 50 μl of H_2O (Jiang et al., 2003), was collected into an equal volume of anticoagulation buffer (Ma and Kanost, 2000). After incubation in a 70°C water bath for 10 min with agitation, the hemolymph sample was chilled on ice and centrifuged at $39,200 \times g$ for 20 min. The supernatant was applied to a reverse phase HPLC column equilibrated with 5% acetonitrile, 0.1% trifluoroacetic acid (TFA). A linear gradient of 5–30% acetonitrile in 0.1% TFA was applied to the C_{18} column at a flow rate of 1.0 ml/min for 30 min with absorbance monitored at 214 nm. Automatically collected fractions (500 μl =tube) were lyophilized and dissolved in 200 μl , 20mM Tris-HCl, pH 7.5. Fractions with PO inhibitory activity were pooled and further separated on the same column using 10% methanol as the mobile phase. The collected fractions were lyophilized, dissolved in 20mM Tris-HCl, pH 7.5, and used for PO inhibitory activity assay and other analyses.

2.5. Determination of PO inhibitory activity

To test the recombinant POI, PO was generated by incubating *M. sexta* proPO (1.0 μl , 0.3 $\mu\text{g}/\mu\text{l}$) with proPO-activating proteinase-1 (PAP-1) (2.0 μl , 30 $\text{ng}/\mu\text{l}$), serine proteinase homolog-1 and -2 (SPHs) (2.0 μl , 50 $\text{ng}/\mu\text{l}$) and 20mM Tris-HCl, pH 7.5 (43 μl) in a 96-well microtiter

plate on ice for 30 min (Jiang et al., 2003). The protein inhibitor (2.0 μ l, 30, 60, 90, 120, 150 and 180 ng/ μ l) was then reacted with PO at 0 °C for 10 min prior to PO activity assay (Jiang et al., 2003). To test the low- M_r inhibitor, *M. sexta* proPO (3.0 μ l; 0.3 μ g/ μ l) or mushroom tyrosinase (3.0 μ l, 100 ng/ μ l) (Sigma) and 20mM Tris-HCl, pH 7.5 (46 μ l), were mixed with 1.0 μ l of the compound at different dilutions. The residual activities were measured using dopamine as a substrate (Jiang et al., 2003) in the presence (for PO) or absence (for mushroom tyrosinase) of 0.2% cetylpyridinium chloride (CPC)

2.6. Trypsin and chymotrypsin digestion of POI

The low- M_r inhibitor (3 μ l) or recombinant POI (6 μ l, 160ng/ μ l) from *E. coli* was incubated with 1 μ l of trypsin or chymotrypsin (1.0mg/ml, Sigma) in a total volume of 30 μ l at 37°C for 8 h. After heating at 100 °C for 5 min to inactivate the proteinase, 10 μ l of the reaction mixtures were separately mixed with 40 μ l of induced larval plasma (1:40 diluted in 20mM Tris-HCl, pH 7.5). Residual PO activity was determined using 100 μ l, 2mM dopamine solution containing 0.2% CPC (Jiang et al., 2003)

2.7. Expression analysis by RT-PCR

The RNA sample (2–4 μ g), oligo(dT) (0.5 μ g) and dNTPs 1 μ l, 10mM each) were mixed with diethylpyrocarbonate-treated H₂O in a final volume of 12 μ l, denatured at 65 °C for 5 min and quickly chilled on ice for 3 min. M-MLV reverse transcriptase (1 μ l, 200U= μ l, Invitrogen), 5 $\times$ buffer (4 μ l), 0.1M dithiothreitol (2 μ l) and RNase OUT (1 μ l, 40U/ μ l, Invitrogen) were added to the denatured RNA sample (12 μ l) for cDNA synthesis at 37 °C for 50 min. The *M. sexta* ribosomal protein S3 mRNA was used as an internal control to normalize the cDNA samples in a PCR using primers 501 (5'-GCCGTTCTTGCCCTGTT-3') and 504 (5'-CGCGAGTTGACTTCGGT-3'). POI cDNA fragment was amplified using j218 (5'-AGAACTTGATAAGGGACG-3') and j219 (5'-ACCTTATTAACCGGAGCCAGACACG-3') under the conditions empirically chosen to avoid saturation. The cycling conditions were 25, 30 or 35 cycles of 94 °C for 30s, 55 °C for 30 s, 72 °C for 30 s. The relative cDNA levels of POI in the normalized samples were determined by 1% agarose gel electrophoresis.

3. Results

3.1. Expression and purification of *M. sexta* POI in *E. coli*

M. sexta POI cDNA was isolated from a male antennal library in an EST project (Robertson et al., 1999). The 572-nucleotide DNA includes a complete open reading frame encoding an 88-residue polypeptide. Following the putative signal peptide, the mature protein is composed of 73 amino acid residues (Fig. 1). While there is no *N*-linked glycosylation site in the sequence, *M. sexta* POI is predicted to be modified at Ser¹¹ (Gupta et al., 1999). Multiple sequence alignment indicated that residues 1–40 of the molecule do not align with its homologs from the other insects. The remaining part of *M. sexta* POI is 33–58% identical in sequence to equivalent regions in the other proteins. This includes six absolutely conserved cysteine residues involved in disulfide bond formation. As determined in *M. domestica* POI (Daquinag et al., 1999), the same disulfide linkage pattern (Cys₁–Cys₄, Cys₂–Cys₅ and Cys₃–Cys₆) may also exist in the other proteins to stabilize a common three-dimensional structure. Among the highly conserved residues (Gly⁴⁷, His⁵³, Asp⁵⁵, Ser⁵⁸, Leu⁶², Tyr⁶⁴, Lys⁶⁷ and Val⁶⁹ in *M. sexta* POI), Tyr⁶⁴ may directly interact with the reactive center of its target enzyme, tyrosinase-type POs. In the housefly POI, this residue was hydroxylated to become dopa (Daquinag et al., 1995)

For functional analyses, we produced *M. sexta* POI as a soluble fusion protein using an *E. coli* expression system. Since the reducing environment of cytoplasm may affect disulfide bond

formation, folding and protein association, we selected pET40 as the expression vector for producing *M. sexta* POI. The fusion partner, DsbC, led the nascent polypeptide to the more oxidative periplasmic space of *E. coli* and catalyzed isomerization of incorrectly paired Cys residues. Osmotic shock was then applied to release the recombinant protein as well as other periplasmic enzymes. Using the S tag and 6×His tag encoded by the plasmid, we detected and purified the soluble fusion protein by affinity chromatography (Fig. 2). After specific proteolytic digestion at the enterokinase cleavage site, the uncleaved protein and tagged fusion partner were removed by Ni²⁺—NTA agarose, whereas the liberated POI was further purified by reverse phase HPLC. From 1L of the *E. coli* culture, we obtained approximately 130 µg of POI. Automated Edman degradation indicated that its first seven amino acid residues (Ser-Pro-Gly-Asn-Leu-Ile-Arg) were the same as the original design. MALDI mass spectrometry showed that the purified protein had a molecular mass of 8197.5 Da, 6.7 units smaller than the theoretical value 8204.3 Da) of the recombinant protein (SPGNLIR ... RCVSGSG). This suggested that the six cysteine residues formed three disulfide bonds and led to the decrease of 6.0 Da. The inhibitory activity of *M. sexta* POI further confirmed the correct Cys pairing and protein folding (Fig. 3).

We further tested whether or not the recombinant protein affects the cleavage activation of proPO. There was little difference in the extent of proPO proteolysis (Fig. 3), suggesting that the reduction of PO activity was caused by inhibition of PO activity rather than proPO activation. Nevertheless, the recombinant protein produced in *E. coli* did not effectively block PO activity: under the experimental conditions, 3.8 µg/ml of POI or a POI:PO molar ratio of ≥6:1 was required to inhibit 50% of the total activity (Fig. 3). The concentration-dependent inhibition fits well with the one-phase exponential decay ($r^2 = 0.97$), suggesting that PO and POI associated loosely.

3.2. Expression and purification of *M. sexta* POI from the insect cells

To examine if the lack of *O*-glycosylation had led to the low inhibitory activity, we produced *M. sexta* POI in baculovirus-infected Sf21 cells. We first incorporated a synthetic DNA fragment encoding honeybee melittin signal peptide into pFH6, a modified pFastBac1 containing a coding region for the 6 × His affinity tag in the carboxyl terminus (Ji et al., 2003). Then, the amplified POI cDNA was inserted to pMHF6, yielding POI/pMHF6 and respective baculovirus. From Sf21 cells infected by the virus, the recombinant POI was successfully secreted to the medium and reached a low concentration (~50 µg=1) at 96h. After affinity chromatography, the active fractions were pooled and further separated by reverse phase HPLC (Fig. 4). The first two protein peaks were pure POI and we obtained approximately 25 µg *M. sexta* POI from 1 L of the conditioned medium.

The molecular mass of POI in peak 1 was determined to be 10,684 Da, larger than the calculated value of the 6 × His tagged protein (8971 Da). The major mass difference of 1713 Da was probably due to *O*-linked glycosylation at Ser¹¹. Consistent with that, *O*-glycosidase treatment of the POI resulted in an increase in the electrophoretic mobility on the SDS-polyacrylamide gel (Fig. 5). There was a reduction in the PO inhibitory activity accompanied by the removal of sugar moiety. Since the activity decrease was not significant and POI from *E. coli* had a high IC₅₀ of 3.8 µg/ml (Fig. 2), the glycosylated POI (presumably very similar to that in *M. sexta* plasma) does not appear to be an effective regulator of PO activity, unless a critical modification is present in the plasma protein but not in the recombinant ones.

3.3. Isolation of a low-Mr POI

Because the housefly POI contained a dopa at the position equivalent to Tyr⁶⁴, we tested if recombinant tyrosine hydroxylase (a kind gift from Dr. Gorman at Kansas State University) could hydroxylate POI and significantly elevate its activity. Mass spectrometry did not show

any mass increase, suggesting that POI was not modified by the hydroxylase. We then attempted but failed to directly isolate POI from the *M. sexta* hemolymph, partly because antibodies against the recombinant protein from *E. coli* did not react well with the plasma protein. Finally, we employed the PO inhibition assay to monitor the purification, hoping that the antibodies would better recognize POI after a few fractionation steps. While the target protein was still undetected, we discovered a strong activity against PO and mushroom tyrosinase (Fig. 6). After we separated the active fraction by SDS-PAGE in Tris-Gly-SDS or Tris-Tricine-SDS buffer, there was no band visualized by Coomassie blue or silver staining. Neither did mass analysis reveal any peak with a molecular mass greater than 500 Da. We suspected that this activity came from a small M_r compound hidden in the matrix peaks. To further characterize the inhibitory activity, we separated the pooled active fraction by reverse phase HPLC on the C18 column using 10% methanol as the mobile phase. While three absorbance peaks were detected, the third one demonstrated a strong inhibitory activity—PO activity decreased from 15.2 to 0.4 U. We further characterized this potent POI by proteolysis: trypsin or chymotrypsin digestion did not abolish its inhibitory activity (Fig. 6). On the contrary, the recombinant POI lost its activity after the same treatment. UV spectroscopy revealed a broad peak with a maximum absorption at 257 nm. Consistent with our speculation, this heat-stable colorless compound did not show a significant absorption at 214 or 280 nm, typical for proteinaceous samples.

3.4. Expression profile analysis of *M. sexta* POI by RT-PCR

In order to test whether or not POI transcription is up-regulated after an immune challenge, we analyzed total RNA samples of hemocytes and fat body from *M. sexta* larvae injected with water or a mixture of microorganisms. POI mRNA was undetected in the hemocyte samples, whereas amplification of the induced fat body cDNA yielded a major PCR product at the expected size. The band intensity was much higher than that of the control (Fig. 7). We also inspected the POI mRNA levels in fat body at different developmental stages and found that the transcripts were present in fat body from day 3, fourth instar. The mRNA level declined in the fifth instar and resurged at the early wandering stage. After a decrease in the rest of wandering stage, POI transcript level gradually increased in the pupae.

4. Discussion

Insect POs are likely involved in melanotic encapsulation, wound healing and hemolymph coagulation. Their proteolytic activation is under strict regulation, mainly by serine proteinase inhibitors of the serpin family (Kanost et al., 2004). Active POs are also regulated by different means (Sugumaran, 2002; Tsukamoto et al., 1992). Based on the sequence similarity, we identified a potential POI from *M. sexta* and produced it in *E. coli* for activity analysis (Fig. 1). To ensure formation of the correct disulfide bonds, we expressed the POI as a fusion protein with DsbC, which acts as a chaperone to improve the folding of proteins with complex disulfide linkages (Jurado et al., 2002; Maskos et al., 2003). We then raised a rabbit polyclonal antiserum against the purified POI that inhibited *M. sexta* PO (Fig. 2 and Fig. 3). However, the antiserum failed to detect the natural protein from *M. sexta*, suggesting that the hemolymph POI was present at a low concentration and/or in a considerably modified form.

Hence, we expressed the POI in *Sf21* cells in order to test the role of glycosylation and to obtain sufficient amount of the glycoprotein for preparing another polyclonal antiserum. The yield of recombinant POI in insect cells was low (25 μ g/l) and the protein differed in glycosylation extent (Fig. 4 and unpublished results). Upon removal of the carbohydrate moiety, the PO inhibitory activity decreased (Fig. 5), implying that the glycosylation enhances the interaction between PO and POI.

At its estimated concentration in the larval hemolymph, even the glycosylated POI is not expected to play a major role in the regulation of PO. This is consistent with the finding that POI knockdown did not lead to a faster or more extensive melanization of Sephadex beads injected to the mosquitoes (Shi et al., 2006). To test if a posttranslational modification (e.g. Try⁶⁴ to dopa) occurring *in vivo* could greatly enhance its inhibitory activity, we attempted but failed to isolate the natural POI from the *M. sexta* hemolymph for structural and activity comparison.

Instead, we isolated a low- M_r compound with potent inhibitory activity against the PO and mushroom tyrosinase (Fig. 6). Based on the initial results, we suggest that this compound contains a phenyl ring which strongly absorbs at 250–260 nm. Further chemical analyses are largely limited by the sample quantity. Despite an unknown structure, the compound represents the first endogenous low- M_r POI isolated from an insect. Previous study showed that POs from Coleopteran and Lepidopteran insects were strongly inhibited by a fungi metabolite (Dowd, 1999). Produced by the entomopathogens *Aspergillus* and *Penicillium* species, kojic acid blocks the PO activities and protects the fungi from the host defense response. In contrast, the small inhibitor described herein perhaps minimizes damage of PO-generated compounds to host tissues/cells.

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References

- Ashida M, Brey PT. Role of the integument in insect defense: prophenoloxidase cascade in the cuticular matrix. *Proc. Natl. Acad. Sci. USA* 1995;92:10698–10702. [PubMed: 11607587]
- Bidla G, Lindgren M, Theopold U, Dushay MS. Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. *Dev. Comp. Immunol* 2005;29:669–679. [PubMed: 15854679]
- Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev* 2004;198:116–126. [PubMed: 15199959]
- Daquinag AC, Nakamura S, Takao T, Shimonishi Y, Tsukamoto T. Primary structure of a potent endogenous dopa-containing inhibitor of phenol oxidase from *Musca domestica*. *Proc. Natl. Acad. Sci. USA* 1995;92:2964–2968. [PubMed: 7708756]
- Daquinag AC, Sato T, Koda H, Takao T, Fukuda M, Shimonishi Y, Tsukamoto T. A novel endogenous inhibitor of phenoloxidase from *Musca domestica* has a cystine motif commonly found in snail and spider toxins. *Biochemistry* 1999;38:2179–2188. [PubMed: 10026302]
- Dowd PF. Relative inhibition of insect phenoloxidase by cyclic fungal metabolites from insect and plant pathogens. *Nat. Toxins* 1999;7:337–341. [PubMed: 11122526]
- Dunn P, Drake D. Fate of bacteria injected into naïve and immunized larvae of the tobacco hornworm, *Manduca sexta*. *J. Invertebr. Pathol* 1983;41:77–85.
- Gupta R, Jung E, Gooley AA, Williams KL, Brunak S, Hansen J. Scanning the available *Dictyostelium discoideum* proteome for O-linked GlcNAc glycosylation sites using neural networks. *Glycobiology* 1999;9:1009–1022. [PubMed: 10521537]
- Ji C, Wang Y, Ross J, Jiang H. Expression and in vitro activation of *Manduca sexta* prophenoloxidase-activating proteinase-2 precursor (proPAP-2) from baculovirus-infected insect cells. *Protein Expression Purif* 2003;29:235–243.
- Jiang H, Wang Y, Yu XQ, 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* 2003;278:3552–3561. [PubMed: 12456683]
- Jurado P, Ritz D, Beckwith J, de Lorenzo V, Fernandez LA. Production of functional single-chain Fv antibodies in the cytoplasm of *Escherichia coli*. *J. Mol. Biol* 2002;320:1–10. [PubMed: 12079330]

- Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol. Rev* 2004;198:97–105. [PubMed: 15199957]
- Kim SR, Yao R, Han Q, Christensen BM, Li J. Identification and molecular characterization of a prophenoloxidase involved in *Aedes aegypti* chorion melanization. *Insect Mol. Biol* 2005;14:185–194. [PubMed: 15796752]
- Li D, Scherfer C, Korayem AM, Zhao Z, Schmidt O, Theopold U. Insect hemolymph clotting: evidence for interaction between the coagulation system and the prophenoloxidase activating cascade. *Insect Biochem. Mol. Biol* 2002;32:919–928. [PubMed: 12110299]
- Lourenco AP, Zufelato MS, Bitondi MMG, Simoes ZLP. Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera*. *Insect Biochem. Mol. Biol* 2005;35:541–552. [PubMed: 15857760]
- Ma C, Kanost MR. A β , 1-3-glucan-recognition protein from an insect, *Manduca sexta* agglutinates microorganisms and activates the phenoloxidase cascade. *J. Biol. Chem* 2000;275:7505–7514. [PubMed: 10713054]
- Maskos K, Huber-Wunderlich M, Glockshuber R. DsbA and DsbC-catalyzed oxidative folding of proteins with complex disulfide bridge patterns in vitro and in vivo. *J. Mol. Biol* 2003;325:495–513. [PubMed: 12498799]
- Nappi AJ, Christensen BM. Melanization and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochem. Mol. Biol* 2005;35:443–459. [PubMed: 15804578]
- Robertson HM, Martos R, Sears CR, Todres EZ, Walden KK, Nardi JB. Diversity of odorant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol* 1999;8:501–518. [PubMed: 10620045]
- Shi L, Li B, Paskewitz SM. Cloning and characterization of a putative inhibitor of melanization from *Anopheles gambiae*. *Insect Mol. Biol* 2006;15:313–320. [PubMed: 16756550]
- Sugumaran M. Unified mechanism for sclerotization of insect cuticle. *Adv. Insect Physiol* 1998;27:229–334.
- Sugumaran M. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigm. Cell Res* 2002;15:2–9.
- Sugumaran M, Nellaiappan K. Characterization of a new phenoloxidase inhibitor from the cuticle of *Manduca sexta*. *Biochem. Biophys. Res. Commun* 2000;268:379–383. [PubMed: 10679212]
- Sugumaran M, Nellaiappan K, Amaratunga C, Cardinale S, Scott T. Insect melanogenesis. III. Metabolon formation in the melanogenic pathway-regulation of phenoloxidase activity by endogenous dopachrome isomerase (decarboxylating) from *Manduca sexta*. *Arch. Biochem. Biophys* 2000;378:393–403. [PubMed: 10860557]
- Tsukamoto T, Ichimaru Y, Kanegae N, Watanabe K, Yamaura I, Katsura Y, Funatsu M. Identification and isolation of endogenous insect phenoloxidase inhibitors. *Biochem. Biophys. Res. Commun* 1992;184:86–92. [PubMed: 1567460]

Glossary

Abbreviation

PO and proPO

phenoloxidase and its precursor

PAP

proPO-activating proteinase

SPH

serine proteinase homolog

POI

phenoloxidase inhibitor

DsbC

	<i>E. coli</i> protein disulfide isomerase
EST	expressed sequence tag
HPLC	high-performance liquid chromatography
MALDI-TOF	matrix-assisted laser desorption ionization-time of flight
IPTG	isopropyl- β -D-thiogalactopyranoside
NTA	nitriloacetic acid
PAGE	polyacrylamide gel electrophoresis
PTU	1-phenyl-2-thiourea
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
CPC	cetylpyridinium chloride

Fig. 1.

M. sexta POI and its comparison with homologous sequences from other insects. (A) cDNA and deduced amino acid sequence. The predicted signal peptide is underlined, and the *O*-linked glycosylation site is marked "■". Shaded Tyr⁶⁴ corresponds to dopa³² in the housefly POI, which is considered to be critical for its activity. (B) Alignment of the disulfide-knotted regions in homologous molecules. Ms, *M. sexta* (BE015616); Bm, *Bombyx mori* (BY939736); Md, *M. domestica* (AAB33998); Ag, *Anopheles gambiae* (CD747521); Aa1 and Aa2, *Aedes aegypti* (EB099073 and EB102275, respectively); He, *Heliconius ethilla* (DT668523.3); Dm1 and Dm2, *Drosophila melanogaster* (BK002735 and BK002734, respectively). Six Cys residues that form three disulfide bonds (Cys₁–Cys₄, Cys₂–Cys₅ and Cys₃–Cys₆) in *M. domestica* POI (Daquinag et al., 1999) are conserved in all of the sequences. The highly similar residues (Gly, His, Asp, Ser, Leu, Tyr, Lys, Val) are shaded and the conserved Tyr is boxed.

Fig. 2.

Isolation of *M. sexta* POI from *E. coli*. (A) SDS-PAGE and immunoblot analysis of the DsbC-POI fusion protein in the periplasmic extract (lane 1), Ni-NTA elution fractions (lane 2) and enterokinase digest (lane 3). Left panel, silver staining; right panel, immunoblot analysis using S-protein antibodies. Positions and sizes of the M_r markers are indicated. The fusion protein and DsbC are marked by arrows. (B) Purification of recombinant POI by reverse phase HPLC on a Bio-Rad Hi-Pore RP-318 column. Flow rate: 1.0 ml/min; buffer A: 0.1% TFA in 5% acetonitrile; buffer B: 0.1% TFA in 95% acetonitrile; gradient: 0–75% B in 75 min; UV detection: 214 nm.

Fig. 3. Inhibition of *M. sexta* PO by recombinant POI from *E. coli*. (A) Concentration-dependent inhibition by the purified protein. (B) Effect of POI on proPO activation. Lane 1, proPO (with a small amount of PO cleaved after prolonged storage); lane 2, PO activated by PAP-3 and SPHs; lane 3, PO activated by PAP-3 and SPHs in the presence of recombinant POI (60 ng/μl).

Fig. 4. Purification of recombinant POI produced in baculovirus-infected *Sf21* cells. The recombinant POI from insect cells were enriched and purified by affinity chromatography on an Ni-NTA column. As described in Fig. 1, eluted proteins were further separated by reverse phase HPLC (A). The gradient was 0–40% B in 40 minutes. (B) Inhibitory activity.

Fig. 5. Detection of *O*-linked glycosylation in *M. sexta* POI from *Sf*21 cells. (A) SDS-PAGE analysis of the recombinant POI before (lane 1) and after (lane 2) deglycosylation. (B) Effect of glycosylation on POI activity. 1, purified POI; 2, purified POI treated with *O*-glycosidase; 3 and 4, *O*-glycosidase and buffer controls.

Fig. 6.

Isolation of a low- M_r PO inhibitor from the larval hemolymph. (A) Elution profile. column: Bio-Rad Hi-Pore reverse phase column RP-318; flow rate: 1.0 ml/min; mobile phase: 10% methanol; UV detection: 214 nm. The activity peak is indicated by underlined bar. (B) Inhibition of mushroom tyrosinase (left axis, ●—●) and *M. sexta* PO (right axis, □--□) by the low- M_r compound. (C) Effect of proteinase treatment on the recombinant POI and low- M_r inhibitor. (D) UV absorption spectrum. The low- M_r inhibitor was scanned between 210 and 300nm on a Beckman DU-520 UV/vis spectrophotometer using 20mM Tris-HCl, pH 7.5, as blank.

Fig. 7.

Expression profiles of *M. sexta* POI in fat body at different immune states or development stages. (A) CF and CH, fat body and hemocytes from the naïve larvae; IF and IH, fat body and hemocytes from the larvae injected with bacteria 24 h before. (B) 4_e, fourth instar day 1; 4_m, fourth instar day 3; 4_l, fourth instar day 5; 5_e, fifth instar day 1; 5_m, fifth instar day 3; 5_l, fifth instar day 5; W_e, W_m and W_l, early, middle and late wandering stages; P_e, P_m and P_l, early, middle and late pupal stage.