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Phenoloxidase in Larvae of *Plodia interpunctella* (Lepidoptera: Pyralidae): Molecular Cloning of the Proenzyme cDNA and Enzyme Activity in Larvae Paralyzed and Parasitized by *Habrobracon hebetor* (Hymenoptera: Braconidae)

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Phenoloxidase (PO) is a major component of the insect immune system. The enzyme is involved in encapsulation and melanization processes as well as wound healing and cuticle sclerotization. PO is present as an inactive proenzyme, prophenoloxidase (PPO), which is activated via a protease cascade. In this study, we have cloned a full-length PPO1 cDNA and a partial PPO2 cDNA from the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) and documented changes in PO activity in larvae paralyzed and parasitized by the ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae). The cDNA for PPO1 is 2,748 bp and encodes a protein of 681 amino acids with a calculated molecular weight of 78,328 and pI of 6.41 containing a conserved proteolytic cleavage site found in other PPOs. *P. interpunctella* PPO1 ranges from 71–78% identical to other known lepidopteran PPO-1 sequences. Percent identity decreases as comparisons are made to PPO-1 of more divergent species in the orders Diptera (*Aa*-48; *As*-49; and *Sb*-60%) and Coleoptera (*Tm*-58; *Hd*-50%). Paralyzation of host larvae of *P. interpunctella* by the idiobiont *H. hebetor* results in an increase in phenoloxidase activity in host hemolymph, a process that may protect the host from microbial infection during self-provisioning by this wasp. Subsequent parasitization by *H. hebetor* larvae causes a decrease in hemolymph PO activity, which suggests that the larval parasitoid may be secreting an immunosuppressant into the host larva during feeding. Arch. Insect Biochem. Physiol. 59:67–79, 2005. Published 2005 Wiley-Liss, Inc.†

KEYWORDS: *Plodia interpunctella*; immune system; phenoloxidase; cDNA; *Habrobracon*; ectoparasitoid

INTRODUCTION

Phenoloxidase (PO), also called tyrosinase, possesses both monophenol monooxygenase activity (E.C. 1.14.18.1) and *o*-diphenoloxidase activity (E.C. 1.10.3.1), and is responsible for initiating the biosynthesis of melanin. PO is associated with three physiologically important biochemical processes in insects and arthropods. These include (1) sclerotization of insect cuticle (Sugumaran, 1998), (2) encapsulation and melanization of foreign organisms (Söderhäll et al., 1990; Ashida and Brey, 1995; Gillespie et al., 1997), and (3) wound healing (Lai-Fook, 1966; Ashida and Brey, 1998).

Although the physiological importance of PO in insect physiology makes it important for study, loss of activity during purification of the labile enzyme and self inactivation have prevented detailed

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The complete nucleotide sequence of *Plodia interpunctella* PPO1 cDNA can be accessed through the NCBI-GenBank database, Accession Number AY665397.

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characterization of insect POs in the past (Sugumaran and Kanost, 1993). However, because PO is present as the inactive zymogen, purification and characterization of the more stable prophenoloxidase (PPO) have been successful. PPO has been purified and at least partially characterized from a number of different insects including the lepidopterans *Bombyx mori* (Ashida, 1971; Yasuhara et al., 1995), *Manduca sexta* (Aso et al., 1985; Hall et al., 1995; Jiang et al., 1997a), *Hyalophora cecropia* (Andersson et al., 1989), and *Galleria mellonella* (Kopácek et al., 1995), the dipterans *Musca domestica* (Hara et al., 1993), *Drosophila melanogaster* (Fujimoto et al., 1993), and *Sarcophaga bullata* (Chase et al., 2000), the coleopterans *Tenebrio molitor* (Heyneman, 1965) and *Holotrichia diomphalia* (Kwon et al., 1997), as well as *Blaberus discoidalis* (Durrant et al., 1993) and *Locusta migratoria* (Cherqui et al., 1996). These PPOs are more similar in amino acid sequence to arthropod hemocyanins than to fungal or mammalian tyrosinases, especially with respect to their putative copper binding sites. Although cDNAs for these PPOs do not encode signal peptides (Kawabata et al., 1995), PPO is thought to be released from hemocytes. Evidence exists that PPOs can be either glycosylated or non-glycosylated (Sugumaran, 2002).

In addition to the purification of these PPOs, cDNAs that encode these enzymes have been cloned and sequenced from *Galleria mellonella* (Li et al., 2002), *Manduca sexta* (Hall et al., 1995; Jiang et al., 1997a), *Bombyx mori* (Kawabata et al., 1995), *Hyphantria cunea* (Park et al., 1997), *Anopheles gambiae* (Jiang et al., 1997b; Lee et al., 1998; Müller et al., 1999), *Aedes aegypti* (Taft et al., 2001), *Sarcophaga bullata* (Chase et al., 2000), *Tenebrio molitor* (Lee et al., 1999), *Holotrichia diomphalia* (Kim et al., 2002), *Armigeris subalbatus* (Cho et al., 1998), and Drosophila melanogaster (Fujimoto et al., 1993; Chase and Sugumaran, 2000).

Fabrick et al. (2003) undertook the first studies on the immune system in the Indianmeal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), including recognition of non-self and activation of the PPO cascade. A β-1,3-glucan recognition protein (PiβGRP) was purified from larval hemolymph and its corresponding cDNA was cloned. Functional properties of recombinant PiβGRP were consistent with other recognition proteins implicated in activation of innate immune responses. Recombinant deletion mutants corresponding to the amino-terminal domain of PiβGRP recognized β-1,3-glucan, LPS, and lipoteichoic acid and subsequently activated the PPO system (Fabrick et al. 2004). The carboxyl-terminal domain of PiβGRP did not activate PPO.

We are using the interaction of the idiobiont ectoparasitoid *Habrobracon hebetor* (Say) with larvae of *P. interpunctella* as a means of characterizing the immune system in this pyralid moth. Previously, we found no observable changes in the composition of hemolymph proteins in paralyzed or parasitized hosts (Baker and Fabrick, 2000). However, the immune system of *P. interpunctella* remains competent after paralyzation by this wasp (Fabrick, unpublished data) and there is evidence for several physiological changes in paralyzed and parasitized host larvae. These changes include evidence for an immunosuppressant secreted by the parasitoid larva as it feeds (Baker and Fabrick, 2002), as well as a significant increase in osmotic pressure in hemolymph from paralyzed hosts (Hartzer et al. unpublished data). In our continuing studies on this host-parasitoid association, a cDNA encoding PPO1 has been cloned from *P. interpunctella* and we demonstrate that host hemolymph PO activity gradually increases in paralyzed larvae but decreases during parasitization.

MATERIALS AND METHODS

Insects

A field strain (El Paso) of *Plodia interpunctella* was collected from infested corn in El Paso, Illinois, in November 1998. Moths were reared in the laboratory on a diet consisting of wheat shorts, cracked wheat, wheat germ, Brewer's yeast, honey, glycerin, and water (2.8: 1.8: 0.24: 0.12: 0.24: 0.24: 0.13, vol/vol). Cultures were maintained at 27°C and 50–55% humidity with a 12:12 h light:dark cycle. Fifth-instar wandering larvae were used for all experiments.

A strain (Miller) of *Habrobracon hebetor* was collected from wheat infested with *P. interpunctella* in Dickinson County, Kansas, in October 1998. The parasitoid was maintained on larvae of *Ephestia kuehniella* at 27°C and 50–55% humidity.

Cloning and Characterization of PPO cDNA From *P. interpunctella*

Insect PPO amino acid sequences were downloaded from GenBank and aligned by using the PRETTY multiple alignment comparison program on the SeqWeb (Version 2) (Accelrys, San Diego, CA) server. Highly conserved regions were considered for degenerate primers. Primer pairs were designed so that initial fragment size was approximately 800 bp. After initial fragments were obtained, gene specific primers were made for 5′ and 3′-RACE. Primer annealing sites within PPO1 are shown in Figure 1.

cDNA Isolation and Sequencing

A *P. interpunctella* larval whole-body cDNA library in λ Uni-Zap XR vector (Stratagene, La Jolla, CA) (Zhu et al., 2000) was screened using primers designed from alignments of known lepidopteran PPO1 and PPO2 cDNA sequences using regions highly conserved but not located in the copper binding sites. Several primer combinations were used to isolate fragments of both PPO1 and PPO2. Successful primer pairs were Pi_PPO1_f3 (5′CC TTACATGTTCAACTACTG3′) and Pi_PPO1_r3 (5′GCCAGCGGTAGAACA-CCGGGT3′), Pi_PPO1_f4 (5′ACCCGGTGTTCTACCGCTGGC3′) and Pi_PPO1_r2 (5′TCN-GGGTACTTGCGGTC3′). PCR products were run on 1.5% agarose gels and bands obtained by successful primer pairing were excised and subcloned into pCR4-TOPO® vector and used to transform OneShot Chemically Competent *Escherichia coli* (Invitrogen, Carlsbad, CA). Plasmid DNA was purified using QIAprep Plasmid Prep Kit (Qiagen, Valencia, CA) and submitted to the Sequencing and Genotyping Facility at Kansas State University, Manhattan, KS. Inserts were sequenced by using T3 and T7 vector primers. Sequence results were used to synthesize additional gene-specific primers for use in 5′ and 3′ RACE.

5′ and 3′-Rapid Amplification of cDNA

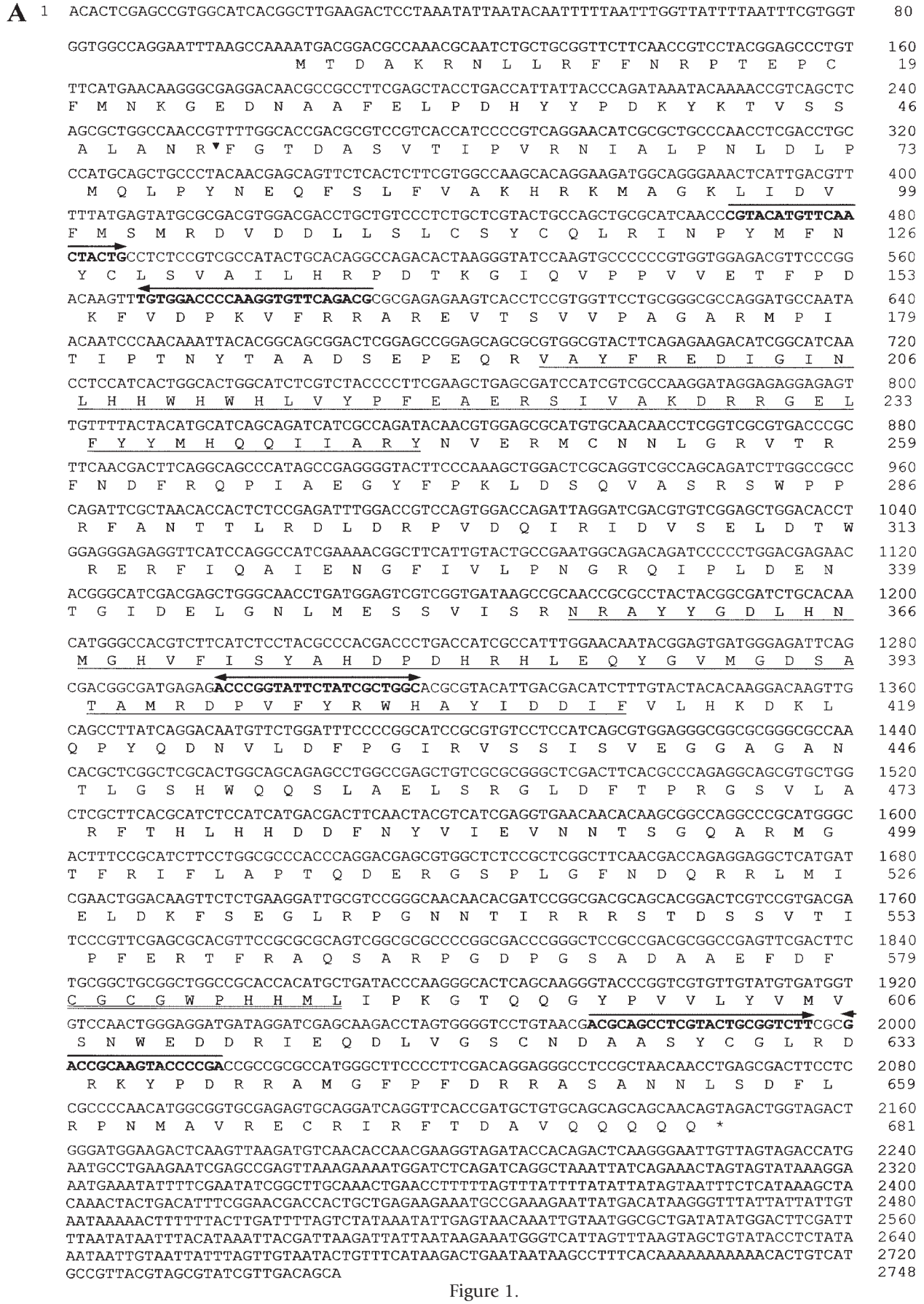
The 5′ and 3′ ends of *P. interpunctella* PPO cDNA were obtained by using RNA ligase mediated rapid amplification (RLM-RACE) of the 5′ and 3′ cDNA ends with the GeneRacer™ kit (Invitrogen) following the protocol of Fabrick et al. (2003). Fifty larvae were homogenized in 4 M guanidine thiocyanate on ice. Total RNA was extracted with phenol: chloroform and precipitated with isopropanol (Titus, 1991). The gene-specific primers, Pi_PPO1_r8 (5′ACGCAGCCTCGTACTGCGGTCTTC 3′) and Pi_PPO1_r7 (5′CGTCTGAACACCTTGGGGTCCACA 3′) were used in conjunction with GeneRacer™ primers specific to the ligated RNA Oligo and Oligo dT. PCR products were subcloned and sequenced as above.

Analysis of Sequence Data

Results from sequencing cloned *P. interpunctella* cDNA were compared to the public sequence database using BLASTX (Altschul et al., 1990). Deduced protein sequences were analyzed using tools from ExPASy Molecular Biology Server of Swiss Institute of Informatics, including Translate, SignalP and Molecular weight/PI calculator. In addition, *N*- and *O*-glycosylation and phosphorylation predictions were made using the NetNGlyc, NetOGlyc, and NetPhos tools found at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/). Percent identity and similarity were calculated with GAP. TreeView (Version 1.6.6) was used to generate a phylogenetic tree from CLUSTALW guide tree data (Page, 1996).

Collection of Hemolymph

Plodia interpunctella larvae were chilled on ice for 30 min and surface sterilized in 95% ethanol. Hemolymph was collected by cutting the 3rd proleg with sterile micro-scissors and drawing the exuded hemolymph into a pipette tip. Two microliters of hemolymph could be collected from single larvae weighing 15–20 mg each. Hemolymph was dispensed into 0.6-ml microcentrifuge tubes, diluted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GGTGGCCAGGAATTTAAGCCAAAATGACGGACGCCAAACGCAATCTGCTGCGGTTCTTCAACCGTCCTACGGAGCCCTGT 160
M T D A K R N L L R F F N R P T E P C 19
TTCATGAACAAGGGCGAGGACAACGCCGCCTTCGAGCTACCTGACCATTATTACCCAGATAAATACAAAACCGTCAGCTC 240
F M N K G E D N A A F E L P D H Y Y P D K Y K T V S S 46
AGCGCTGGCCAACCGTTTTGGCACCGACGCGTCCGTCACCATCCCCGTCAGGAACATCGCGCTGCCCAACCTCGACCTGC 320
A L A N R F G T D A S V T I P V R N I A L P N L D L P 73
CCATGCAGCTGCCCTACAACGAGCAGTTCTCACTCTTCGTGGCCAAGCACAGGAAGATGGCAGGGAAACTCATTGACGTT 400
M Q L P Y N E Q F S L F V A K H R K M A G K L I D V 99
TTTATGAGTATGCGCGACGTGGACGACCTGCTGTCCCTCTGCTCGTACTGCCAGCTGCGCATCAACCCGTACATGTTCAA 480
F M S M R D V D D L L S L C S Y C Q L R I N P Y M F N 126
CTACTGCCTCTCCGTCGCCATACTGCACAGGCCAGACACTAAGGGTATCCAAGTGCCCCCCGTGGTGGAGACGTTCCCGG 560
Y C L S V A I L H R P D T K G I Q V P P V V E T F P D 153
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K F V D P K V F R R A R E V T S V V P A G A R M P I 179
ACAATCCCAACAAATTACACGGCAGCGGACTCGGAGCCGGAGCAGCGCGTGGCGTACTTCAGAGAAGACATCGGCATCAA 720
T I P T N Y T A A D S E P E Q R V A Y F R E D I G I N 206
CCTCCATCACTGGCACTGGCATCTCGTCTACCCCTTCGAAGCTGAGCGATCCATCGTCGCCAAGGATAGGAGAGGAGAGT 800
L H H W H W H L V Y P F E A E R S I V A K D R R G E L 233
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F Y Y M H Q Q I I A R Y N V E R M C N N L G R V T R 259
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T G I D E L G N L M E S S V I S R N R A Y Y G D L H N 366
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T A M R D P V F Y R W H A Y I D D I F V L H K D K L 419
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Q P Y Q D N V L D F P G I R V S S I S V E G G A G A N 446
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E L D K F S E G L R P G N N T I R R R S T D S S V T I 553
TCCCGTTCGAGCGCACGTTCCGCGCGCAGTCGGCGCGCCCCGGCGACCCGGGCTCCGCCGACGCGGCCGAGTTCGACTTC 1840
P F E R T F R A Q S A R P G D P G S A D A A E F D F 579
TGCGGCTGCGGCTGGCCGCACCACATGCTGATACCCAAGGGCACTCAGCAAGGGTACCCGGTCGTGTTGTATGTGATGGT 1920
C G C G W P H H M L I P K G T Q Q G Y P V V L Y V M V 606
GTCCAACTGGGAGGATGATAGGATCGAGCAAGACCTAGTGGGGTCCTGTAACGACGCAGCCTCGTACTGCGGTCTTCGCG 2000
S N W E D D R I E Q D L V G S C N D A A S Y C G L R D 633
ACCGCAAGTACCCCGACCGCCGCGCCATGGGCTTCCCCTTCGACAGGAGGGCCTCCGCTAACAACCTGAGCGACTTCCTC 2080
R K Y P D R R A M G F P F D R R A S A N N L S D F L 659
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R P N M A V R E C R I R F T D A V Q Q Q Q Q * 681
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Figure 1.

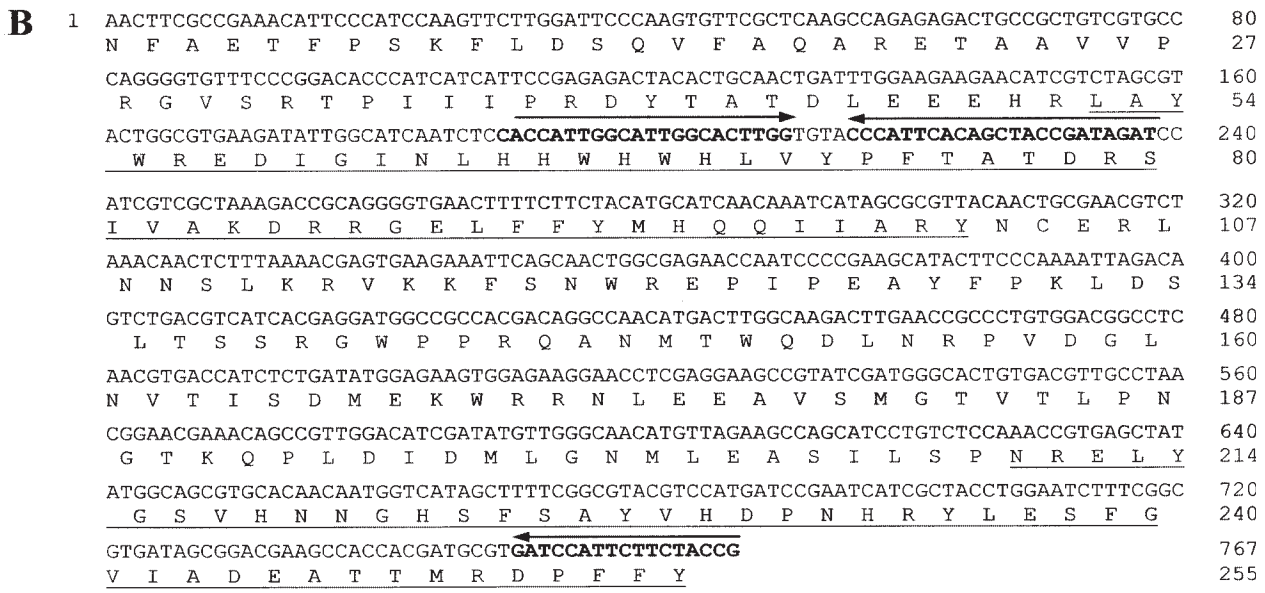


Fig. 1. Nucleotide and the deduced amino acid sequence of *P. interpunctella* PPOs. **A:** The PPO1 cDNA nucleotide sequence (1–2,748) is shown above the deduced amino acid sequence (1–681). The proteolytic cleavage site (RF) is marked with an arrowhead. The putative thiol ester site is double underlined. Amino acid sequences underlined with a single line indicate the copper-binding regions. The termination codon TAG is marked with an asterisk. Primers were designed based on alignments of other lepidopteran PPO1 sequences. Regions expressing highly conserved sequence were used to design primers. The primer pairs PiPPO1f3 (468–486) × PiPPO1r3 (1,296–1,316) and PiPPO1f4 (1,296–1,316) × PiPPO1r2 (2,000–2,016) were successful in obtaining initial sequence fragments from the full body *P. interpunctella* cDNA library. PiPPO1r7 (568–591) and PiPPO1f8 (1,974–1,996) are gene-specific primers used in 3′ and 5′-RACE, respectively. **B:** The partial cDNA nucleotide sequence of PPO2 is shown above the deduced amino acid sequence. Amino acid sequences underlined with a single line indicate the copper-binding regions. Primers were designed based on alignments of other lepidopteran PPO2 sequences. The primer pair PiPPO2f1 × PiPPO2r3 was successful in obtaining the region that encodes the copper-binding sites. A gene-specific primer (PiPPO2r6) was used to obtain additional sequence closer to the 5′-end.

1:25 (vol:vol) in distilled water, and centrifuged at 10,000*g* for 5 min at 4°C to pellet hemocytes. Cell-free plasma was used in PO activity assays. Protein concentration was determined by using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) with a BSA standard.

Analysis of PO Activity

Phenoloxidase activity was assayed by using a method modified from Hall et al. (1995) and Jiang and Kanost (1997). Diluted plasma (25 µl) was added to designated wells of a microplate containing 50 µl 100 mM phosphate buffer pH 7.0 and either 5 µl H_2O or 5 µl elicitor (*Micrococcus lysodeikticus*, Sigma, 5 × 10^6 cells). After 20 min, substrate (20 µl of 10 mM dopamine, 2 mM final substrate concentration) was added and phenoloxidase activity (mOD/min) was determined by measuring absorbance at 490 nm at 5-min intervals for 30 min at 30°C using a Bio-Tek EL-340, 96-well microplate reader. Reaction rates were obtained with KC^3 software (Bio-Tek Instruments, Inc., Winooski, VT).

Effect of Paralyzation on PO Activity

Fifth instar *P. interpunctella* were placed in a 100 × 15 mm plastic Petri dish and *H. hebetor* females were placed in the dish at a ratio of approximately 1 wasp per 10 *Plodia* larvae. All larvae were generally stung and paralyzed within 3 h. All paralyzed

A

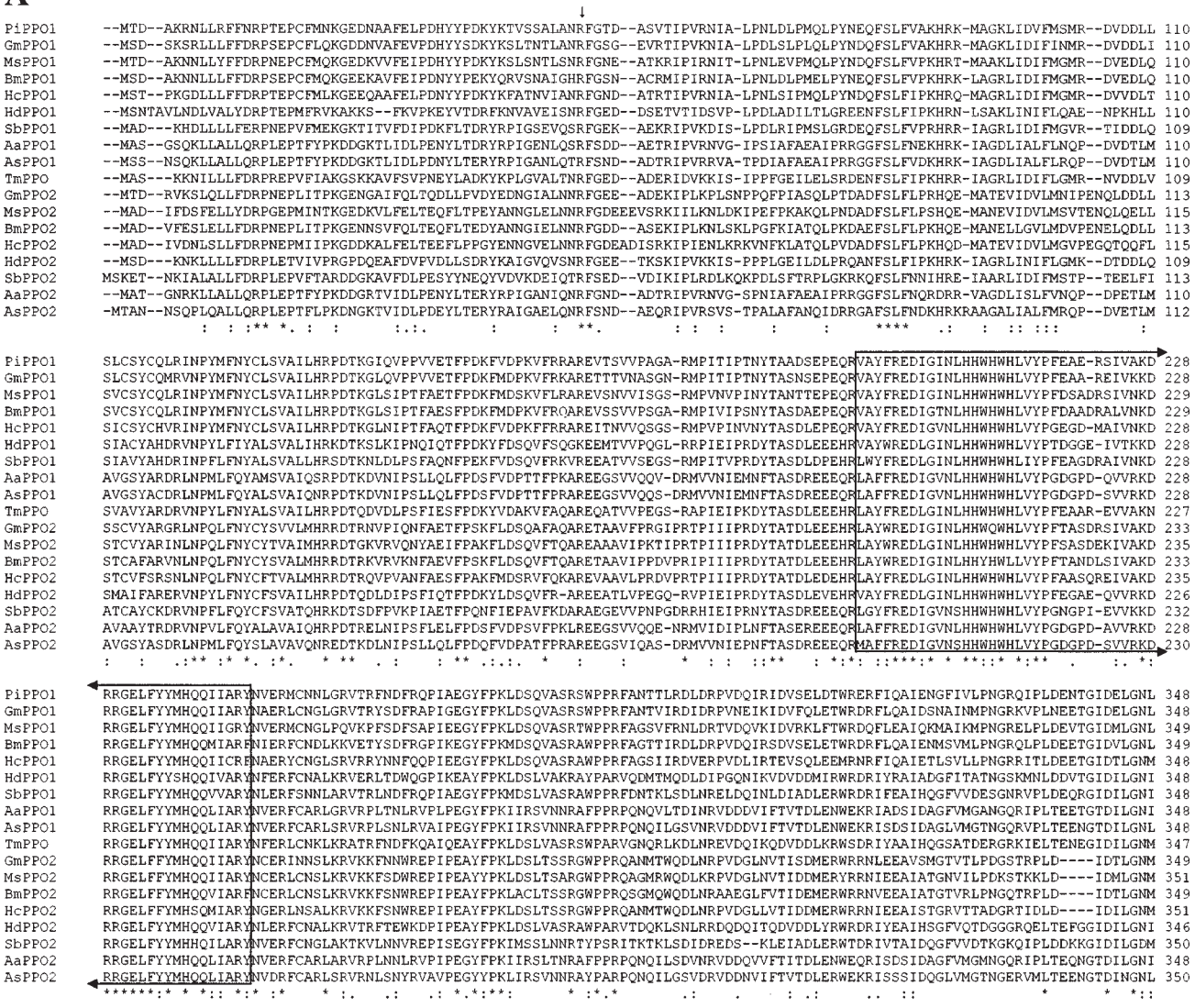
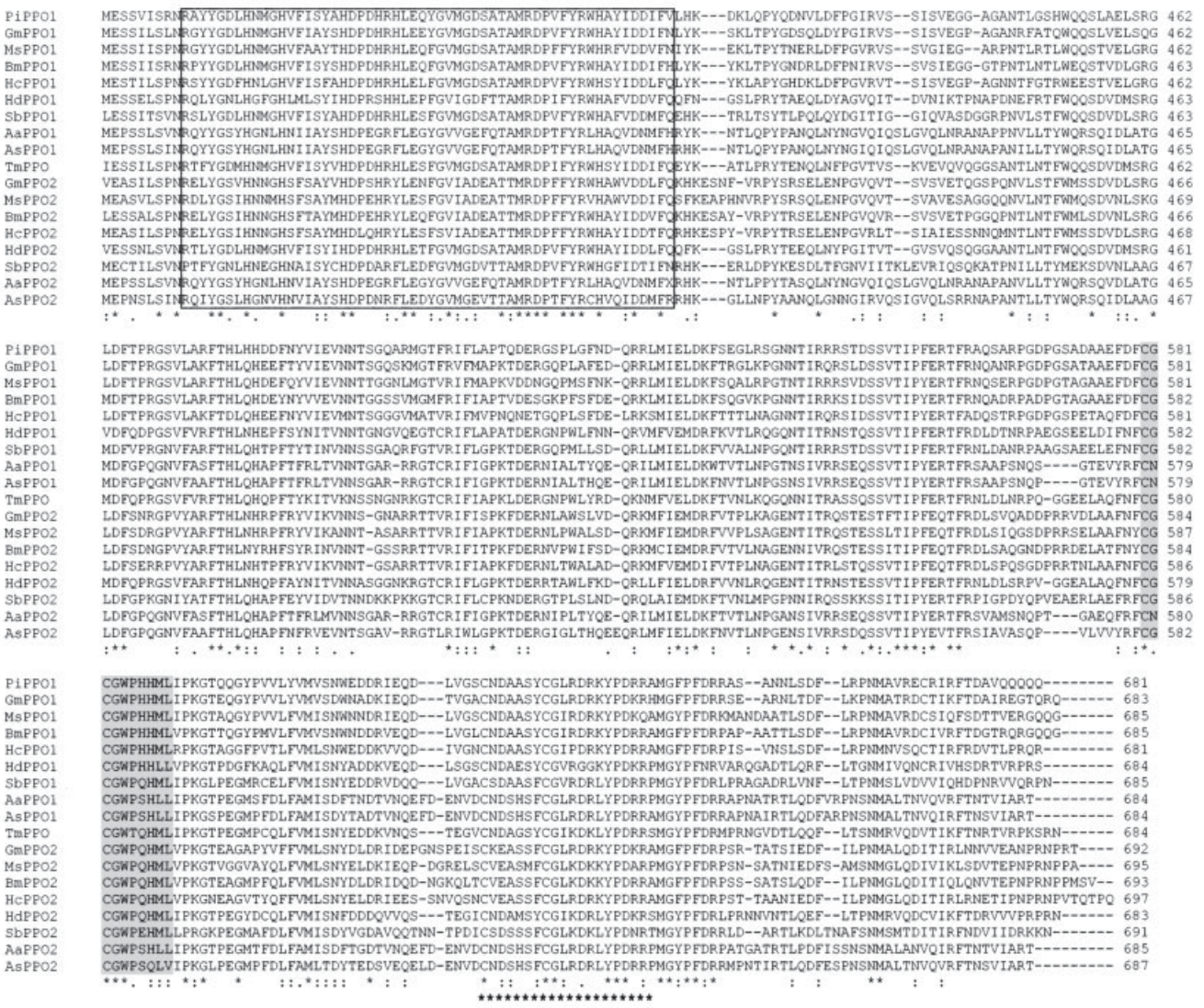
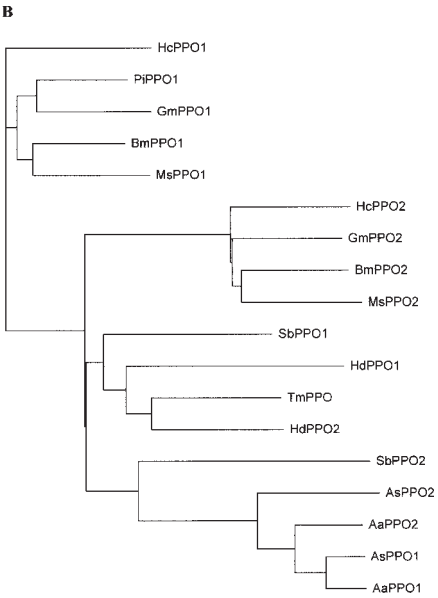


Fig. 2. Sequence alignment and phylogenetic tree showing relationship between *P. interpunctella* PPO1 with other prophenoloxidase family members. **A:** The CLUSTALW multiple sequence alignment program was used to align the *P. interpunctella* PPO1 amino acid sequence with 17 other insect protein sequences found within the GenBank database. Identical residues are marked with an asterisk (*), strongly conserved residues are marked with a colon, and weakly conserved residues are marked with a period. A vertical arrow marks the RF proteolytic cleavage site activating the proenzyme. Copper binding regions are outlined in boxes. Putative thiol region is shaded in gray. Bold asterisks mark the conserved C terminal region found in many invertebrates. **B:** Phylogenetic distances were obtained from a CLUSTALW alignment of the 18 sequences and a tree was constructed using TreeView. The aligned sequences are as follows: PiPPO1, *P. interpunctella* PPO1; GmPPO1, *G. mellonella* PPO1; MsPPO1, *M. sexta* PPO1; BmPPO1, *B. mori* PPO1; HcPPO1, *H. cunea* PPO1; HdPPO1, *H. diomphalia* PPO1;TmPPO, *T. molitor* PPO; SbPPO1, *S. Bullata* PPO1; AaPPO1, *A. aegypti* PPO1; AsPPO1, *A. subalbatus* PPO1; GmPPO2, *G. mellonella* PPO2; MsPPO2, *M. sexta* PPO2; BmPPO2, *B. mori* PPO2; HcPPO2, *H. cunea* PPO2; HdPPO2, *H. diomphalia* PPO2; SbPPO2, *S. Bullata* PPO2; AaPPO2, *A. aegypti* PPO2; AsPPO2, *A. subalbatus* PPO2.





larvae were removed to a clean dish and held at room temperature. Hemolymph was collected at 1-, 2-, 4-, and 7-day intervals. The experiment was replicated with 5 separate trials consisting of 9–12 larvae for each time period per trial.

Effect of Parasitization on PO Activity

Plodia and *Habrobracon* were placed together in Petri dishes as in the paralysis assays. After 24 h, half of the paralyzed *Plodia* larvae were removed and the remaining larvae were left as hosts for oviposition by *H. hebetor*. After egg hatch, individual parasitoid larvae were transferred (1 parasitoid/host larva) to new, previously paralyzed hosts from the

original cohort. The parasitoid larvae were allowed to feed for either 1 or 2 days. Egg hatch generally occurred on the 3rd or 4th day of paralyzation so the feeding times for 1 and 2 days corresponded to 5 and 6 days paralyzation. Hemolymph was collected from host larvae 1 and 2 days after parasitization and assayed for PO activity.

RESULTS

Properties of *P. interpunctella* PPO cDNAs

PCR amplification with degenerate primers was used to isolate partial clones of two PPOs from a whole-body cDNA library in λ Uni-Zap XR vector. 3′- and 5'-RACE were used to obtain the missing ends of the PPO1 sequence. The complete sequence for PPO1 is shown in Figure 1A and the partial sequence of PPO2 is shown in Figure 1B along with their deduced amino acid sequence. The cDNA for PPO1 is 2,748 bp and encodes a protein of 681 amino acids with a calculated molecular weight of 78,328 and pI of 6.41. There was no evidence of a secretion signal peptide for PPO1.

The deduced amino acid sequence of PPO1 was compared to other PPOs in GenBank (Fig. 2A). Alignments show that the copper binding sites are conserved in all of the proteins. Also, the PPOs contained a conserved proteolytic cleavage site (RF) as well as a previously characterized thiol ester site CGCGWPQHML (Hall et al, 1995) that has a His substitution for Gln in the C-terminal region. The copper binding region was present in PPO2.

Sequence Comparisons

Sequence comparisons were made between *P. interpunctella* and several other insect species in the orders Lepidoptera, Diptera, and Coleoptera. *P. interpunctella* PPO1 ranges from 71–78% identical to other known lepidopteran PPO-1 sequences. As Lepidoptera, Coleoptera, and Diptera evolved and became more divergent, their PPOs are less conserved and percent identity decreases. Compared to *P. interpunctella* PPO1, sequence identities ranged from 48–60% in the Diptera and 50-58% in the Coleoptera. A phylogram prepared in TreeView (Fig. 2B) shows the clear divergence of PPO1s and PPO2s among the Lepidoptera.

Effects of Paralyzation and Parasitization on PO Activity in *P. interpunctella*

Phenoloxidase activity levels among control, paralyzed, and parasitized larvae were compared by using 5 separate trials with 9–12 larvae per time period per trial. Analysis of phenoloxidase activity of paralyzed larvae showed a gradual increase beginning 24 h after stinging (Fig. 3). Initial PO activity levels were 0.3 ± 0.06 mOD/min for control larvae. PO activity increased about 10-fold after 7 days, up to a level of 3.0 ± 0.13 mOD/min. There was no detectable PO activity in venom gland homogenates (Hartzer, 2004) so the increase in PO activity in paralyzed larvae did not result from injection of the enzyme into the host larva during envenomization.

Addition of parasitoid larvae to previously paralyzed hosts resulted in an immediate reduction in host PO activity (Fig. 3). After 1 day of feeding, the PO activity of host larvae declined by approximately 50% to 1.2 ± 0.03 mOD/min. PO activity continued to decrease during parasitization. Because of the reduction in hemolymph volume caused by feeding of the parasitoid larvae, assays could not conducted after 3 days of parasitization.

DISCUSSION

Several prophenoloxidases have been purified and characterized in insects (Hall et al., 1995; Kawabata et al., 1995; Jiang et al. 1997a; Cho et al., 1998; Ashida and Brey, 1998, Chase et al., 2000). Two PPOs have been characterized from several Lepidoptera including *Manduca sexta*, *Bombyx mori*, *Galleria mellonella*, and *Hyphantria cunea*, and in the case of some Diptera many more PPOs have been characterized. In this work, a full-length cDNA of PPO1 and a partial sequence of PPO2 from *P. interpunctella* were identified.

Plodia interpunctella PPO1 ranges from 71–78% identical to other known lepidopteran PPO-1 sequences. Although sequence similarity is high for

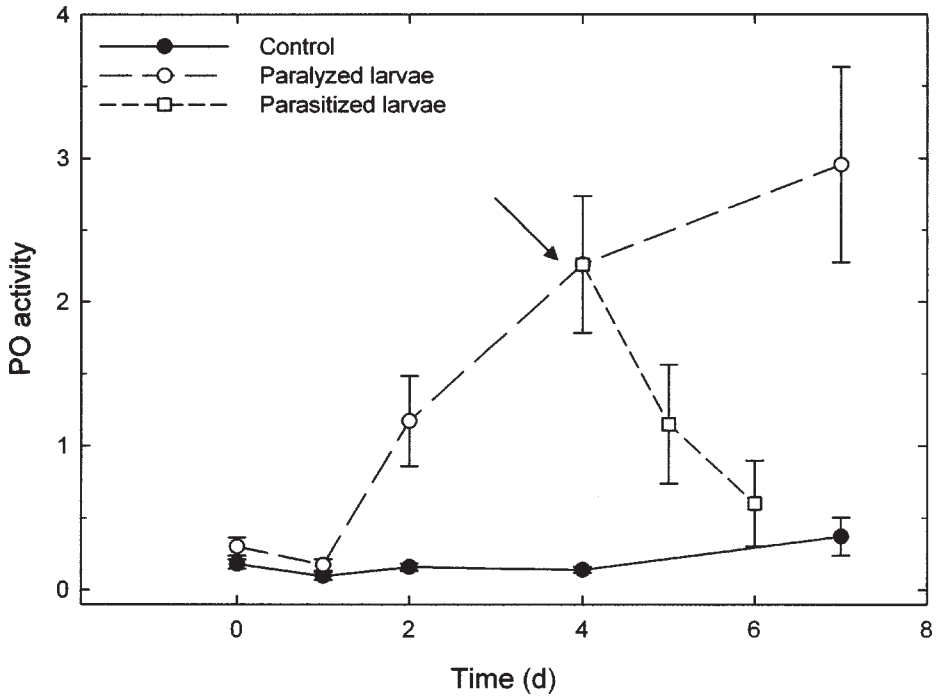


Fig. 3. Effects of paralyzation and parasitization on PO activity in hemolymph of *P. interpunctella*. PO activity was measured in hemolymph of *P. interpunctella* larvae that had been either paralyzed or paralyzed and subsequently parasitized. An arrow indicates the beginning of parasitization by the placement of *H. hebetor* larvae onto paralyzed hosts. PO activity is defined as the mean increase in absorbance (mOD/min) at 490 nm in the reaction mixture during a 30-min time interval. Each point is the mean (S.E.) of 5 cohorts of 9–12 larvae per cohort from different *Plodia* generations.

all PPOs when compared to other lepidopteran PPO1s, the identity is exceptionally high. *Plodia* PPO1 has a higher similarity to PPO1 from *Galleria* and other insects than it does with its own PPO2. This characteristic has been displayed for many insects containing more than one PPO. However, only a single PPO has been identified in the cockroach, *Blaberus discoidalis* (Durrant et al., 1993), the African migratory locust, *Locusta migratoria* (Cherqui et al., 1996), and the crayfish, *Pacifastacus leniusculus* (Aspán and Söderhäll, 1991).

The full-length *P. interpunctella* PPO1 cDNA possesses high-sequence similarity to other insect PPOs, especially other lepidopterans, including *M. sexta*, *B. mori*, *H. cunea*, and *G. mellonella* as well as the dipterans *S. bullata*, *A. subalbatus*, and *A. aegypti* and the two beetles *T. molitor* and *H. diomphalia*. All of these PPOs contain the RF proteolytic cleavage site, two copper-binding regions, and a putative C-terminus thiol ester. Additionally, a conserved C-terminal site, similar to one found in arylphorins (Brumester and Scheller, 1996), was present. Insect PPOs in general, lack a signal peptide and are thus unlikely to have N-glycosylation associated with protein movement across the cell membrane. However, in other Lepidoptera, phenoloxidase from *G. mellonella* has been shown to be a glycoprotein (Kopácek et al., 1995), but the PPOs of *M. sexta* (Jiang et al., 1997a) and *B. mori* (Yasuhara et al., 1995) are not. *Plodia interpunctella* PPO1 does not have a signal peptide but showed two possible N-glycosylation sites. The PPOs of *S. bullata* have several N-glycosylation sites but did not stain for sugars with Schiff's reagent or bind Concanavalin A suggesting a lack of glycosylation (Chase et al., 2000). However, because *P. interpunctella* is most closely related to *G. mellonella*, i.e., both are pyralid moths, with their PPO sequences showing the highest identity, it is possible that *Plodia* PPOs are glycoproteins as well. Studies of binding affinity of PiPPO1 for Concanavalin A could be used to clarify this question.

Insect PPOs share similarities with hemolymph hexameric storage proteins, and with Arthropod hemocyanins, which are copper-containing hexamers (Fujimoto et al, 1993; Hall et al, 1995; Kawabata et al., 1995). The three-dimensional structure of hemocyanin from the spiny lobster, *Panulirus interruptus*, shows the hexamer to be formed from trimers of tightly associated dimers, with the major contacts between the subunits containing the copper-binding sites. As was the case

in *M. sexta* (Jiang et al., 1997a), *Plodia* PPO1 was 29% identical to *P. interruptus* hemocyanin with a higher identity corresponding to the copper-binding regions. Until the structure of an insect PPO is available, the arthropod hemocyanins will have to serve as a model for the enzyme.

Physiological effects of parasitization by ectoparasitoids include changes in host hemocyte morphology and viability, as well as suppression of immune reactions, including phagocytosis and encapsulation (Richards and Edwards, 1999, 2000a, 2002). Richards and Edwards (2000b) also demonstrated that although envenomization of the host by the wasp adult did not suppress PO activity, feeding by the ectoparasitoid larvae significantly reduced hemolymph activity of this enzyme. Suppression of PO activity has also been shown to occur in parasitism of lepidopteran hosts by entomopathogenic nematodes (Yokoo et al., 1992). These results contrast with those of Sroka and Vinson (1978) who found no suppression of hemolymph PO activity in response to endoparasitoid attack. Although attack by the idiobiont ectoparasitoid *H. hebetor* results in fatal envenomization of *P. interpunctella* larvae, we provide evidence in the current study that both the adult wasp and parasitoid larva can induce humoral immune responses in the host larvae.

When *H. hebetor* adults sting the host larvae, changes occur at both the physiological and immunological levels. Physiologically, and most obvious, is the paralysis of the host. This paralysis is due to the presynaptic blockage of glutamatergic excitatory transmission, resulting in loss of neuromuscular control (Spanjer et al., 1977; Piek et al., 1982; reviewed in Piek, 1990) . At the immunological level, venom from *H. hebetor*, either directly or indirectly, causes an increase in PO activity in host hemolymph with time after stinging. The increase in PO activity may be beneficial to *H. hebetor*, as increased levels of PO may help suppress microbial infection during the time interval between stinging and host utilization, and thus preserve the nutritional value of the paralyzed larva as a subsequent food source for the parasitoid (Hagstrum and Smittle, 1978; Hagstrum, 1983).

The larval parasitoid of *H. hebetor* demonstrates a much different effect on the *P. interpunctella* host than does the adult wasp. While both adults and larvae have an effect on the humoral response of the host, our evidence suggests that the larval parasitoid may suppress PO activity in host hemolymph in a manner similar to that described for *Lacanobia oleracea* parasitized by *Eulophus pennicornis* (Richards and Edwards, 2000b). The reduced PO activity may be the result of an immunosuppressant secreted into the host by the parasitoid larva. Similarly, a diffusible immunosuppressant may also be responsible for the unmelanized region surrounding the feeding site on surrogate host larvae (see fig. 2 from Baker and Fabrick, 2002). Whether the same factor is responsible for both phenomena is not known. Finally, our observations that host hemolymph leaches through the parasitoid feeding sites (Hartzer, 2004) support the hypothesis that ectoparasitoids secrete an anticoagulant or some factor inhibiting the wound healing process as suggested by Strand and Pech (1995). We also observed that host hemolymph did not leach through feeding sites surrounded by melanized rings.

Because phenoloxidase is only one enzyme in a complex cascade, exactly where the adult venom or larval factors exert their action remains to be determined. There are several possible reasons for changes in the level of PO activity in paralyzed and parasitized *P. interpunctella* host larvae. The increase in PO activity during paralysis may be caused by lysis of hemocytes in the host leading to increased levels of PPO in the hemolymph. However, because the enzyme is in the pro-form, no direct increase in PO activity would be observed simply from lysis of hemocytes. Increased PO activity may be the result of activation of enzymes upstream from PPO such as prophenoloxidase activating proteinases (PAPs), or venom components themselves may cause PPO to cleave to its active form. As for suppression of PO activity by the parasitoid larva, it is more likely that the secreted factor(s) affect the regulatory mechanisms of the PPO cascade, i.e., through the action of inhibitors or activating enzymes, or perhaps as early as the recognition of non-self.

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LITERATURE CITED

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410.

Andersson K, Sun SC, Boman HG, Steiner H. 1989. Purification of the prophenoloxidase from *Hyalophora cecropia* and four proteins involved in its activation. Insect Biochem 19:629–637.

Ashida M. 1971. Purification and characterization of prephenoloxidase from hemolymph of the silkworm, *Bombyx mori*. Arch Biochem Biophys 144:749–762.

Ashida M, Brey PT. 1995. Role of the integument in insect defense: pro-phenol oxidase cascade in the cuticular matrix. Proc Natl Acad Sci USA 92:10698–10702.

Ashida M, Brey PT. 1998. Recent advances in research on the insect prophenoloxidase cascade. In: Brey PT, Hultmark D, editors. Molecular mechanisms of immune responses in insects. London: Chapman & Hall. p 135–172.

Aso Y, Kramer KJ, Hopkins TL, Lookhart GL. 1985. Characterization of hemolymph protyrosinase and a cuticular activator from *Manduca sexta* (L). Insect Biochem 15:9–17.

Aspán A, Söderhäll K. 1991. Purification of prophenoloxidase from crayfish blood cells, and its activation by an endogenous serine proteinase. Insect Biochem 21:363–373.

Baker JE, Fabrick JA. 2000. Host hemolymph proteins and protein digestion in larval *Habrobracon hebetor* (Hymenoptera: Braconidae). Insect Biochem Mol Biol 30:937–946.

Baker JE, Fabrick JA. 2002. Unusual responses of Indianmeal moth larvae (Lepidoptera: Pyralidae) to envenomation and parasitization by a braconid ectoparasitoid. J Entomol Sci 37:370–374.

Brumester T, Scheller K. 1996. Common origin of arthropod tyrosinase, arthropod hemocyanin, insect hexamerin and dipteran arylphorin receptors. J Mol Evol 42:713–728.

Chase MR, Sugumaran M. 2000. Genomic and cDNA sequences of prophenoloxidase from *Drosophila melanogaster*. Adv Exp Med Biol 484:349–362.

Chase MR, Raina K, Bruno J, Sugumaran M. 2000. Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*. Insect Biochem Mol Biol 30:953–967.

Cherqui A, Duvic B, Brehelin M. 1996. Purification and characterization of prophenoloxidase from the hemolymph of *Locusta migratoria*. Arch Insect Biochem Physiol 32:225–235.

Cho WL, Liu H, Lee CH, Kuo CC, Chang TY, Liu CT, Chen CC. 1998. Molecular cloning, characterization and tissue expression of prophenoloxidase cDNA from the mosquito, *Armigeres subalbatus* inoculated with *Dirogilaria immitis* microfilariae. Insect Mol Biol 7:31–40.

Durrant H, Ratcliffe NA, Hipkin CR, Aspán A, Söderhäll K. 1993. Purification of the prophenoloxidase enzyme from hemocytes of the cockroach *Blaberus discoidalis*. Biochem J 289:87–91.

Fabrick JA, Baker JE, Kanost MR. 2003. cDNA cloning, purification, properties, and function of a β-1,3-glucan recognition protein from a pyralid moth, *Plodia interpunctella*. Insect Biochem Mol Biol 33:579–594.

Fabrick JA, Baker JE, Kanost MR. 2004. Innate immunity in a pyralid moth: functional evaluation of domains from a β-1,3-glucan recognition protein. J Biol Chem 279:26605–26611.

Fujimoto K, Masuda K, Asada N, Ohnishi E. 1993. Purification and characterization of prophenoloxidase from the pupae of *Drosophila melanogaster*. J Biochem (Tokyo) 113:285–291.

Gillespie JP, Kanost MR, Trenczek T. 1997. Biological mediators of insect immunity. Annu Rev Entomol 42:611–643.

Hagstrum DW. 1983. Self-provisioning with paralyzed hosts and age, density, and concealment of hosts as factors influencing parasitization of *Ephestia cautella* (Walker) (Lepi-

doptera: Pyralidae) by *Bracon hebetor* Say (Hymenoptera: Braconidae). Environ Entomol 12:1727–1732.

Hagstrum DW, Smittle BJ. 1978. Host utilization by *Bracon hebetor*. Environ Entomol 7:596–600.

Hall M, Scott M, Sugumaran M, Söderhäll K, Law JH. 1995. Proenzyme of *Manduca sexta* phenoloxidase: purification, activation, substrate specificity of the active enzyme and molecular cloning. Proc Natl Acad Sci USA 92:7764–7768.

Hara T, Miyoshi T, Funatsu M. 1993. Comparative studies on larval and pupal phenoloxidases of the housefly, *Musca domestica*. Comp Biochem Physiol 106B:287–292.

Hartzer KL. 2004. Host-parasitoid interaction: biological, biochemical and molecular apsects of the prophenoloxidase cascade in *Plodia interpunctella* (Lepidoptera: Pyralidae) parasitized by *Habobracon hebetor* (Hymenoptera: Braconidae). M.S. Thesis, Department of Entomology, Kansas State University, Manhattan, KS 66502.

Heyneman RA. 1965. Final purification of a latent phenoloxidase with mono- and diphenoloxidase from *Tenebrio molitor*. Biochem Biophys Res Commun 21:162–169.

Jiang H, Kanost MR. 1997. Characterization and functional analysis of 12 naturally occurring reactive site variants of serpin-1 from *Manduca sexta*. J Biol Chem 272:1082–1087.

Jiang H, Wang Y, Ma C, Kanost MR. 1997a. Subunit composition of prophenoloxidase from *Manduca sexta*: molecular cloning of subunit ProPo-P1. Insect Biochem Mol Biol 27:835–850.

Jiang H, Wang Y, Korochkina SE, Beneš H, Kanost MR. 1997b. Molecular cloning of cDNA for two pro-phenol oxidase subunits from the malaria vector, *Anopheles gambiae*. Insect Biochem Mol Biol 27:693–699.

Kawabata T, Yasuhara Y, Ochia, M, Matsuura S, Ashida M. 1995. Molecular cloning of insect prophenoloxidase: A copper containing protein homologous to arthropod hemocyanin. Proc Natl Acad Sci USA 92:7774–7778.

Kim MS, Baek MJ, Lee MH, Park JW, Lee SY, Soderhall K, Lee BL. 2002. A new easter-type serine protease cleaves a masquerade-like protein during prophenoloxidase activation in *Holotrichia diomphalia* larvae. J Biol Chem 277:39999–40004.

Kopácek P, Weise C, Gotz P. 1995. The prophenoloxidase from the wax moth *Galleria mellonella*: Purification and characterization of proenzyme. Insect Biochem Mol Biol 25: 1081–1091.

Kwon TH, Lee SY, Lee JH, Choi JS, Kawabata SI, Iwanaga S, Lee BL. 1997. Purification and characterization of prophenoloxidase from the hemolymph of coleopteran insect, *Holitrichia diomphalia*. Mol Cells 7:90–97.

Lai-Fook J. 1966. The repair of wounds in the integument of insects. J Insect Physiol 12:195–226.

Lee HS, Cho MY, Lee KM, Kwon TH, Homma K, Natori S, Lee BL. 1999. The pro-phenoloxidase of coleopteran insect, *Tenebrio molitor*, larvae was activated during cell clump/cell adhesion of insect cellular defense reactions. FEBS Lett 444:255–259.

Lee W-J, Ahmed A, della Torre A, Kobayashi A, Ashida M, Brey PT. 1998. Molecular cloning and chromosomal localization of a prophenoloxidase cDNA from the malaria vector *Anopheles gambiae*. Insect Mol Biol 7:41–50.

Li D, Scherfer C, Korayem AM, Zhao Z, Schmidt O, Theopold U. 2002. Insect hemolymph clotting: evidence for interaction between the coagulation system and the prophenoloxidase activating cascade. Insect Biochem Mol Biol 32:919–928.

Müller HM, Dimopoulos G, Blass G, Kafatos FC. 1999. A hemocyte-like cell line established from malaria vector *Anopheles gambiae* expresses six phenoloxidase genes. J Biol Chem 274:11727–11735.

Page RDM. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358.

Park DS, ShinW, Kim MG, Park SS, Lee WJ, Brey PT, Park HY. 1997. Isolation and characterization of the cDNAs encoding the prophenoloxidase of fall webworm, *Hyphantria cunea*. Insect Biochem Mol Biol 27:983–992.

Piek T. 1990. Neurotoxins from venoms of the Hymenoptera: twenty-five years of research in Amsterdam. Comp Biochem Physiol C 96:223–233.

Piek T, Veenendaa RL, Mantel P. 1982. The pharmacology of *Microbracon* venom. Comp Biochem Physiol C 72:303–309.

Richards EH, Edwards JP. 1999. Parasitization of *Lacanobia oleracea* (Lepidoptera: Noctuidae) by the ectoparasitic wasp, *Eulophus pennicornis*: effects of parasitization, venom

and starvation on host haemocytes. J Insect Physiol 45:1073–1083.

Richards EH, Edwards JP. 2000a. Parasitization of *Lacanobia oleracea* (Lepidoptera) by the ectoparasitic wasp, *Eulophus pennicornis*, suppresses haemocyte-mediated recognition of non-self and phagocytosis. J Insect Physiol 46:1–11.

Richards EH, Edwards JP. 2000b. Parasitism of *Lacanobia oleracea* (Lepidoptera) by the ectoparasitoid, *Eulophus pennicornis*, is associated with a reduction in host haemolymph phenoloxidase activity. Comp Biochem Physiol B 127:289–298.

Richards EH, Edwards JP. 2002. Parasitism of *Lacanobia oleracea* (Lepidoptera) by the ectoparasitic wasp, *Eulophus pennicornis*, disrupts the cytoskeleton of host haemocytes and suppresses encapsulation in vivo. Arch Insect Biochem Physiol 49:108–124.

Söderhäll K, Aspán A, Duvic B. 1990. The ProPO system and associated proteins. Role in cellular communication in arthropods. Res Immunol 141:896–907.

Spanjer W, Grosu L, Piek T. 1977. Two different paralysing preparations obtained from a homogenate of the wasp *Microbracon hebetor* (Say). Toxicon 15:413–421.

Sroka P, Vinson SB. 1978. Phenoloxidase activity in the haemolymph of parasitized and unparasitized *Heliothis virescens*. Insect Biochem 8:399–402.

Strand MR, Pech LL. 1995. Immunological basis for compatibility in parasitoid-host relationships. Annu Rev Entomol 40:31–56.

Sugumaran M. 1998. Unified mechanism for sclerotization of insect cuticle. Adv Insect Physiol 27:229–334.

Sugumaran M. 2002. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigment Cell Res 15:2–9.

Sugumaran M, Kanost MR. 1993. Regulation of insect hemolymph phenoloxidases. In: Beckage NE, Thompson SN, Federici BA, editors. Parasites and pathogens of insects, Vol. 1. Parasites. San Diego: Academic Press Inc. p 317–342.

Taft AS, Chen CC, Li J, Christensen BM. 2001. Molecular cloning of two prophenoloxidase genes from the mosquito *Aedes aegypti*. Insect Mol Biol 10:97–103.

Titus D. 1991. Promega protocols and application guide, 2nd ed. Madison, WI: Promega Corporation. p 125–130.

Yasuhara Y, Koizumi Y, Katagiri C, Ashida M. 1995. Reexamination of properties of prophenoloxidase isolated from larval hemolymph of the silk worm *Bombyx mori*. Arch Biochem Biophys 320:14–23.

Yokoo S, Tojo S, Ishibashi N. 1992. Suppression of the prophenoloxidase cascade in the larval haemolymph of the turnip moth, *Agrotis segetum* by an entomopathogenic nematode, *Steinernema carpocapsae* and its symbiotic bacterium. J Insect Physiol 38:915–924.

Zhu YC, Kramer KJ, Dowdy AK, Baker JE. 2000. Trypsinogen-like cDNAs and quantitative analysis of mRNA levels from the Indianmeal moth, *Plodia interpunctella*. Insect Biochem Mol Biol 30:1027–1035.