Oxidative conjugation of catechols with proteins in insect skeletal systems


Abstract — Cuticle sclerotization or tanning is a vital process that occurs during each stage of insect development to harden and stabilize the newly secreted exoskeleton. The structural polymers protein and chitin make up the bulk of the cuticle, and chemical interactions between these biopolymers with quinonoid tanning agents are largely responsible for the physical properties of the mature exoskeleton. The oxidative conjugation of catechols with cuticular proteins plays an important role in this metabolism. The main hypothesis for cuticle sclerotization involves the formation of adducts and cross-links between nucleophilic imidazole nitrogens of histidyl residues in the proteins and electrophilic ring or side-chain carbons of ortho-quinones and para-quinone methides derived from the catechols, N-acetyldopamine, N-beta-alanyldopamine, and 3,4-dihydroxyphenylethanol. C–N and C–O linkages between these quinone tanning agents and proteins in cuticles from a variety of insects from several orders have been elucidated. cDNAs for both the tyrosinase and laccase types of phenoloxidases that catalyze the cross-linking reactions have been isolated and sequenced. The sequences of laccase cDNAs from two insect species were more similar to fungal laccases than to those from plants. These results provide insights into how insects use structural proteins, catechols, and oxidative enzymes to form catechol–amino acid adducts during sclerotization. Published by Elsevier Science Ltd.

The o-quinone and p-quinone methide metabolites derived from their catechol precursors by the action of phenoloxidases represent a class of reactive intermediates that can lead to a variety of effects in vivo, including exoskeletal hardening and stabilization, cytotoxicity, immunotoxicity, and carcinogenesis depending on the type of quinonoid and its modifiers as well as the physiological system of interest.1 For hardening and stabilization, insects utilize N-acetyldopamines and other catecholic compounds and the activation of their aromatic rings and side chains by tyrosinases and laccases for nucleophilic addition reactions with proteins.2 This chemistry optimizes the mechanical and chemical properties of exoskeletons, so that species can survive and adapt to their environments. The newly secreted cuticle must be selectively stiffened and hardened by sclerotization processes to provide the necessary mechanical properties for locomotion and protection of each developmental stage. Sclerotization involves in part the formation of covalent bonds between the quinone derivatives of three catechols, N-acetyldopamine (NADA), N-beta-alanyldopamine (NBAD), and 3,4-dihydroxyphenylethanol (DOPET), and side-chain functional groups of histidine and possibly other amino acid residues of cuticular proteins.2–5 During sclerotization as catechols and/or quinone metabolites replace water and interact with the structural proteins by forming several types of covalent conjugates, the cuticle becomes increasingly dehydrated, dense, hydrophobic, and insoluble.

Although insects as a group produce a wide variety of structurally diverse cuticles that differ in chemical and physical properties according to their functional adaptations, little is known about the molecular organization of any of these because of the complexity of their structures and their resistance to degradation for structural analysis. Acid hydrolysates of sclerotized cuticles have yielded products that indicate the types of catechol–protein interactions involved in stabilization.2–5 Arterenone (3, 4-dihydroxyphenylketone) has been shown to represent NADA dimers and oligomers involving benzodioxin-type linkages in cuticle by acting as longer cross-links or filler materials.3 Norepinephrine derivatives represent NADA and NBAD bonded to proteins by their side-chain beta-carbons (C-7) through oxygen.
linkages. Histidyl-dopamine (DA) and histidyl-DOPET adducts represent acid-stable linkages arising from NADA, NBAD, and DOPET being covalently bonded to the ring nitrogens of histidine by the ring and side-chain carbons of the catechols.\textsuperscript{4,5}

The objectives of this study were to (1) compare the types of covalent interactions between catechol tanning agents and proteins in a wide variety of sclerotized cuticles from insects in several orders, and (2) investigate the molecular biology of the enzymes that catalyze the oxidative conjugation of catechols in the cuticle sclerotization process.

### 1. Results and Discussion

#### 1.1. Catechols and their adducts in cuticles

Mass spectrometry was the primary tool used to characterize the structures of catecholic compounds recovered from hydrolysates of cuticles from 14 species representing seven orders of insects.\textsuperscript{5} Arterenone was the major catecholic product obtained from 18 of 21 cuticles that were hydrolyzed (Table 1). Fig. 1 shows the HPLC profile of catechol-containing compounds released by hydrolysis of *Manduca sexta* (tobacco hornworm) pupal exuviae and *Melanoplus sanguinipes* fifth instar larval exuviae. Arterenone (peak \#3, [M]+ 168, data not shown) was particularly high in the light colored NADA-sclerotized cuticles of the grasshopper, but low in the hard brown NBAD-sclerotized pupal cuticle of the tobacco hornworm. It probably was derived from benzodioxin-type catecholic oligomeric and dimeric cross-links, as well as filler material in the predominately NADA-sclerotized cuticles.\textsuperscript{3} Also identified in the hydrolysates was dopamine (peak \#6, Fig. 1, [M]+ 154). Dopamine in the cuticle hydrolysates was derived apparently from any ester-type linkages to ring oxygens and also from free or non-covalently bonded NBAD and NADA that accumulate to relatively high levels in fully sclerotized cuticles.\textsuperscript{5} A norepinephrine (NE)-phenol adduct (peak \#7, [M]+ 245) apparently was formed during the hydrolysis because of the presence of 5\% phenol that was included as an antioxidant and represents catecholic \(\beta\)-carbon (C-7) adducts that had been bonded weakly through C–O linkages such as esters to amino acid residues in the cuticular proteins. Histidyl-DA (peaks \#1, 2, [M]+ 307) and histidyl-DOPET (peaks \#4, 5, [M]+ 308) adducts also were released during hydrolysis, and these represent acid-stable ring and side-chain C–N linkages between catechols and histidine residues in cuticular proteins. Either the 6 (ring)- or the 7 (\(\beta\))-carbon of the catechol was coupled with the N-1 of histidine.

Arterenone was predominant in exuviae from cockroaches (Blattaria), grasshoppers (Orthoptera), dragonflies (Odonata), cicadas (Homoptera), and larval and adult cuticles of Lepidoptera. The dark brown pupal exuvial cuticle of *M. sexta*, sclerotized primarily by NBAD quinones, was particularly low in arterenone and, therefore, NBAD oligomer cross-links, but it was higher in the NE-phenol adduct, which represents C–O bonds between the catechol side-chain and amino acids. Sclerotized cuticles from Coleoptera and the puparia of Diptera also had relatively low amounts of arterenone and higher amounts of the acid stable C–N bonds in histidyl-DA and histidyl-DOPET adducts. Cuticles sclerotized by both NADA and NBAD, such as those of cockroaches, also had higher levels of the histidyl-DA and DOPET adducts as well as arterenone. Relatively large amounts of acid-stable histidyl-DA and histidyl-DOPET adducts as well as lower levels of arterenone were present in the hydrolysates of brown NBAD-sclerotized pupal exuviae of the tobacco hornworm. Therefore, flexible cuticles sclerotized by an NADA quinonoid-mediated mechanism appeared to be stiffened primarily by NADA oligomeric cross-links and C–O catechol protein bonds. On the other hand, less flexible brown cuticles stiffened by an NBAD quinonoid-mediated mechanism apparently were stabilized by more of the C–N acid-stable bonds between histidyl residues and the catechols DOPET and DA that arises from NBAD.

Acid-labile O-bonded catecholic compounds, which gave rise to arterenone, dopamine and norepinephrine, predominated in all sclerotized cuticles analyzed over the acid-stable N-bonded catecholic compounds, which yielded the histidyl-catechol adducts (Table 1). However, the relative amounts of C–O and C–N bonds were nearly equal in the NBAD-mediated sclerotized cuticles of certain cockroaches; pupal exuviae of *M. sexta*; puparial cuticles of Diptera; and exuviae of larval, pupal, and adult cuticles of Coleoptera. The NADA-mediated sclerotized cuticles typically had much higher relative amounts of C–O to C–N bonds as shown in Orthoptera, Odonata (larval exuviae), Homoptera, and larval head capsules and wings of Lepidoptera.

The main source of the C–O linkages were the NADA dimers and oligomers and minor amounts arose from catechol \(\beta\)-carbon linkages to amino acids. However, NBAD-sclerotized cuticles as mentioned above had nearly equal sources of C–O bonds. The C–N bonds were mainly catechol \(\beta\)-carbons (C-7) in the side chain linked to histidyl residues. In puparial cuticles, the C–N bonds were nearly equal between catechol ring and side-chain links to histidyl residues.

#### 1.2. Laccases and phenoloxidases in insects

The initial step in the sclerotization process involves formation of highly reactive tanning agents by the oxidation of catechols or o-diphenols to their corresponding electrophilic o-benzoquinones. This reaction is catalyzed by copper oxidases in the phenoloxidase group and requires molecular oxygen as a hydrogen acceptor. Two classes of phenoloxidases, known as tyrosinases and laccases, are present in insect integuments.\textsuperscript{2}

Tyrosinases catalyze both the hydroxylation of monophenols and the oxidation of o-diphenols to o-benzoquinones (Fig. 2, reactions 2 and 6) but do not oxidize p-diphenols. Tyrosinase cDNAs have been isolated from several insect species, including two isoforms of this enzyme from *M. sexta*.\textsuperscript{7,8} The sequences of insect tyrosinases are related to arthropod hemocyanins, which use a
Table 1. Catechol-containing compounds in acid hydrolysates of cuticles from seven orders of insects

<table>
<thead>
<tr>
<th>Order</th>
<th>Species</th>
<th>Stage/Cuticle-type</th>
<th>Relative amounts (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td></td>
<td></td>
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<td>7-I</td>
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<tr>
<td>Blattaria</td>
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<td>Coleoptera</td>
<td>Ceratitis capitata</td>
<td>Puparial exuviae</td>
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<sup>a</sup> 7-I=7-(N-1)-histidyldopamine, 6-I=6-(N-1)-histidyldopamine, 7-II=7-(N-1)-histidyl-DOPET, 6-II=6-(N-1)-histidyl-DOPET, ART=arterenone, DA=dopamine, NE=norepinephrine-phenol adduct. ND=none detected. Quantified by HPLC-DAD (280 nm).

Figure 1. HPLC analyses of catechol-containing compounds released by hydrolysis of *Manduca sexta* pupal exuviae and *Melanoplus sanguinipes* fifth instar larval exuviae in 6 M HCl at 110°C for 24 h. 1: 7-(N-1)-histidyld-DA, 2: 6-(N-1)-histidyld-DA, 3: arterenone, 4: 7-(N-1)-histidyl-DOPET, 5: 6-(N-1)-histidyl-DOPET, 6: DA, 7: NE-phenol adduct.
Figure 2. Proposed pathway for protein cross-linking during sclerotization of the pupal cuticle of Manduca sexta. Catechols (N-B-alanyldopamine (NBAD) or 3, 4-dihydroxyphenylethanol (DOPET)) are oxidized by phenoloxidases (tyrosinase and laccase), generating o-quinones and p-quinone methide derivatives (reactions 1 and 2) that serve as electrophilic agents for cuticular proteins. Laccases and quinone isomerases convert the o-quinones to p-quinone methides (3). Histidyl residues in cuticular proteins serve as nucleophiles and undergo Michael addition reactions with either the 5 (ring)- or 7 (β)-carbon atom of the quinonoid intermediates, yielding the 5- or 7-monoadducts of catechol and protein (4 and 5). Additional reactions at the 2-, 6-, and 8-carbons of the catechol also are possible, but to date no mono- or di-adducts bonded at those positions have been detected in pupal cuticle. The phenoloxidases then catalyze a second round of oxidation of the catechol moiety (6 and 7), which is followed by another nucleophilic addition of cuticular proteins to produce 5,7-diadducts (8 and 9). The final product is a supramolecular structure of cuticular proteins cross-linked by C–N linkages with catechols. Legend: P = cuticular proteins with histidyl residues; Q = o-quinones; QM = p-quinone methides; R = –NHCOCH2CH2NH3 (NBAD), –OH (DOPET).
conserved copper-binding site for O₂ transport rather than oxidative reactions.⁹

Tyrosinase is present in insect hemolymph as an inactive zymogen that is activated through a specific proteolytic cleavage by a serine protease.¹⁰ In M. sexta, protyrosinase is synthesized by hemocytes known as oenocytoids.⁵ The tyrosinases isolated from the integument (the outer layer of the insect, comprising the epidermis and the cuticle) and hemolymph of M. sexta have similar physical and catalytic properties,¹⁰–¹² and antiserum to the hemolymph tyrosinase detects proteins of the expected size in extracts of prepupal integument (Fig. 3). Prophenoloxidases 1 (pPO1) and 2 (pPO2) had apparent molecular masses of 78.9 and 79.8 kDa, respectively.¹³ These results suggested that the tyrosinases in integument might be the same two isoforms (pPO1) and (pPO2) that are related. The two sequences have highly significant similarity (25–30% identities of amino acid sequences) to two fungal laccases and a lower degree of similarity to two plant laccases. BLAST (Basic Local Alignment Search Tool, see Table 2) computer searches showed that the insect sequences have highly significant similarity (25–30% identities of amino acid sequences) to two fungal laccases and a lower degree of similarity to two plant laccases (Table 2). Residues conserved in fungal and plant laccases are also present in the insect sequences, including regions containing residues that interact with copper ions in the enzyme’s active site. The two Manduca proteins, in particular, are related remarkably to laccases from microbial and plant sources. Further work is needed to investigate the regulation of insect laccase gene expression during molting cycles and to characterize the enzymatic activity of insect laccases.

Laccase activity in insect cuticles coincides with periods of sclerotization in many species.² The laccase from M. sexta integument is an insoluble enzyme that can be released by proteolysis and exhibits its highest activity towards NBAD, the natural tanning precursor.² It oxidizes both p- and o-diphenols to quinones and also catalyzes the β-hydroxylation of o-diphenols, probably via a quinone methide-type of intermediate (Fig. 2, reactions 1 and 7). This latter intermediate can lead to catechol side-chain adducts, whereas the quinone leads to ring adducts, both of which have been isolated from M. sexta pupal cuticle.¹⁵ Laccase preparations also appear to catalyze the isomerization of o-quinones to p-quinone methides, which then are β-hydroxylated in aqueous solution (Fig. 2, reaction 3)¹⁵ (unpublished data). Laccase may have a major function in the oxidation of catechols that are used for the formation of adducts to the side-chain β-carbon (C-7). However, even though laccase is thought to be very important for the sclerotization of insect cuticle, no insect laccase has been well characterized at the molecular level.

Amino acid sequences of various fungal and plant laccases available in the Genbank database are approximately 30% identical, with regions of much higher identity near the histidine residues that chelate copper ions. We have used a polymerase chain reaction (PCR) approach to isolate cDNAs for putative laccases from M. sexta and from a mosquito, Anopheles gambiae. Alignments of amino acid sequences of fungal and plant laccases were used to identify conserved regions for designing primers, and use of such primers in PCR experiments resulted in amplification of fragments that encode sequences related to laccases known from other sources. BLAST (Basic Local Alignment Search Tool, see Table 2) computer searches showed that the insect sequences have highly significant similarity (25–30% identities of amino acid sequences) to two fungal laccases and a lower degree of similarity to two plant laccases (Table 2). Residues conserved in fungal and plant laccases are also present in the insect sequences, including regions containing residues that interact with copper ions in the enzyme’s active site. The two Manduca proteins, in particular, are related remarkably to laccases from microbial and plant sources. Further work is needed to investigate the regulation of insect laccase gene expression during molting cycles and to characterize the enzymatic activity of insect laccases.

Table 2. Results of BLASTP comparisons of putative insect laccases with fungal and plant laccases

<table>
<thead>
<tr>
<th>Fungal or plant source³</th>
<th>E value⁴ for sequence of</th>
<th>Manduca-1</th>
<th>Manduca-2</th>
<th>Anopheles</th>
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<tr>
<td>Populus balsamifera (Y13771)</td>
<td>7e⁻¹⁶</td>
<td>1e⁻¹⁶</td>
<td>2.9e⁻²</td>
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</table>

² The Expect (E) value is a parameter that describes the number of BLAST hits with the same score expected by chance when searching a database.²²

³ The databank accession numbers are given in parentheses.
2. Concluding remarks

Analysis of catechol–amino acid conjugates and other catechol products present in acid hydrolysates of sclerotized cuticles provided correlations between kinds of catechol–protein covalent interactions and different types of cuticles from a wide variety of insect species. As part of this process, a pathway for protein cross-linking by C–N bond formation during sclerotization of insect cuticles was proposed (Fig. 2). This pathway is consistent with the hypothesis that acid-stable C–N bonds arise between histidyl residues of cuticular proteins and both ring and side-chain carbons of three sclerotization precursors, NADA, NBAD, and DOPET. This cross-linking is catalyzed by phenoloxidases such as laccase and tyrosinase. Analysis of the hydrolysis products of sclerotized cuticles of insects from several orders showed correlations between the relative amounts of catechol–protein adducts bonded by C–N and C–O linkages to the tanning precursors NADA, NBAD, and DOPET. Lighter colored NADA–sclerotized cuticles had relatively high amounts of NADA dimers and oligomers as stabilizing agents and lower amounts of the highly stable C–N bonds. Conversely, the darker colored NBAD–sclerotized cuticles had lower quantities of the oligomeric cross-linkages, which may arise from the oxidation of either NADA or NBAD, and higher levels of the catechol–histidyl C–N bonds. C–O bonds predominated over C–N bonds in all cuticles analyzed but were present in much larger amounts in the NADA-sclerotized cuticles. Generally, the side-chain carbons of catechols were bonded more frequently to amino acid residues of protein through either C–O or C–N links than the ring carbons. Solid-state NMR has provided critical new evidence for the types of catechol–protein and catechol–catechol conjugates in sclerotized cuticle, and it has been confirmed by the analysis of acid hydrolysates. Pathway for C–O cross-link formation is less understood and will be the focus of future investigations. Another long-term goal of this research is to use a combination of recombinant phenoloxidases, cuticular structural proteins, and cross-linking agent precursors in an attempt to model cuticle formation in vitro.

Oxidative conjugation of catechols not only plays a critical role in cuticle physiology, but it also may play an important role in the immune response of insects to microbial pathogens. When an insect’s immune system recognizes a foreign target, one response is the activation of phenoloxidase to produce phenoloxidase, which can hydroxylate tyrosine and oxidize catechols to quinones. These quinones then react and form melanotic layers to encapsulate the foreign target. However, the details of this process are still unknown.

The recent discovery of catechol–thiol adducts in hemolymph of bacteria-challenged insects (unpublished data) suggests that phaemolamin also may be produced as part of the response to infection. We have conducted some preliminary studies on the possible roles of catechols in the insect’s immune response. Formaldehyde-treated bacterial cells (Escherichia coli) were injected into lepidopteran larvae. After one day, hemolymph samples were collected, and the catechol content was determined. When compared with hemolymph collected from insects injected only with water, the concentrations of DA and NBAD were much higher in insects injected with the bacterium. More interestingly, substantial levels of at least three thioether adducts of catechols also were present in the samples from bacterium-injected larvae. Based on their spectroscopic properties, two of these adducts were identified as monosubstituted aryl thiethers, whereas the third was a disubstituted aryl thioether adduct. Thus, not only C–N and C–O adducts but also C–S adducts were utilized by insects in skeletal and immunological systems. Very recently, Kim et al. reported that injection of bacteria into coleopteran larvae also induced the synthesis of NBAD in the hemolymph, as well as several unidentified catechols. However, many of the details underlying the utilization of oxidative conjugation of these catechols in not only the immune responses of many species of insects but also the process of cuticle sclerotization remain to be determined.

3. Experimental

3.1. Isolation of cuticle catechols and adducts

Washed and air-dried cuticles and exuviae were ground in dry ice and lyophilized. The powder (0.25 g) was heated for 24 h at 110°C in 5 ml of 6 M HCl containing 5% phenol in vacuo in a 20-mL hydrolysis tube. Catechols were removed selectively from the hydrolysates by alumina adsorption. After the pH of the hydrolysate was adjusted to pH 8.9 with saturated tris base, EDTA (~1 g) and alumina (~2 g) were added to the solution, which was stirred for 15 min. After centrifugation, the supernatant was collected and subjected to alumina adsorption a second time. The two alumina fractions were pooled and washed with deionized water, and the catechols were desorbed by washing two times with 2 mL 1 M HOAc for 15 min. The HOAc fractions were pooled and concentrated under N₂ for high performance liquid chromatographic (HPLC) analysis. Catechols and adducts were separated using the Spherisorb column and then subjected to chromatography on the Prodigy column if they were not homogeneous after the first Spherisorb chromatography.

3.2. HPLC

A Hewlett-Packard 1050 Series HPLC interfaced with a diode array detector was used and controlled by a HPLC3D Chemstation. Eluents were monitored at 280 nm, the approximate λ_max for catechols and their amino acid adducts. A binary mobile phase was used: solvent A: 0.15 M formic acid and 0.03 M ammonium formate (pH 3.0); solvent B: methanol and aqueous 0.3 M formic acid plus 0.06 M ammonium formate (1/1, v/v). Spherisorb 5 ODS-2 (250x21.2 mm) and Prodigy ODS-2-PREP (10 micron, 250x10 mm) columns were used (Phenomenex, Torrance, CA). At a flow rate of 4 mL/min for the Spherisorb column, 100% of solvent A for 10 min was followed by a linear gradient to 100% solvent B from 10–40 min. The Prodigy column used a flow rate of 2 mL/min, 100% solvent A for 5 min followed by a linear gradient to 100% B from 5–20 min.
3.3. Mass spectrometry

The structures of catechol–histidine adducts previously isolated from the pupal exuviae of Manduca sexta also were analyzed further by mass spectroscopy techniques to further characterize their structures.4,5 Spectra were obtained using either a tandem quadrupole Sciex API III (PE/SCIEX, Thronhill, Ontario, Canada) or a quadrupole ion trap spectrometer (LCQ, Finnigan MAT, San Jose, CA). Samples were infused via a 50 micron-i. d. fused silica transfer line using a Harvard Apparatus pump at a flow rate of 0.4–2 μL/min. Positive ion ESMS and ESMS-MS spectra were acquired using orifice voltages from 25 to 11 V. The interface temperature was 52°C. For tandem mass spectrometry (MS–MS), precursor ions were selected with the first quadrupole (Q1) for collision-induced dissociation (CID) by argon in the second quadrupole (Q2). The third quadrupole (Q3) was scanned with a mass step of 0.1–0.2 μl and 1 ms/step. Mass resolution of Q1 was adjusted to produce a parent ion envelope of 0.7–1.5 mass units wide at the peak half height. Resolution of Q3 varied from −0.4 to 1 mass unit in width at the peak half height. Parameters were sufficient to obtain a valley of 1 to 18% between peaks 1 μl apart. The heated desolvation capillary of the LCQ spectrometer was held at 200°C with the electron multiplier set to 1 kV. The relative collision energy (RCE) was adjusted to obtain diagnostic fragment peaks. The RCE varied from 0 to 5 V peak-to-peak resonance excitation RF voltage.5

High resolution fast atom bombardment (FAB) spectra were obtained with a 6 keV Xe beam and DMSO–thiglycerol (1:1) plus 1% trifluoroacetic acid matrix on a JEOL HX-110 sector instrument at a resolution of ~7500 with a 10% valley. Polyethyleneglycol with a mass distribution peaking at m/z 300 (PEG 300) was used as an internal calibration standard.5

3.4. Characterization of insect phenoloxidases and their cDNAs

Prophenoloxidase was purified from hemolymph of fifth instar M. sexta larvae as described.14 Integument samples were isolated by dissecting day-2 fifth instar larvae or prepupal insects (wandering day 5). Abdominal integument was cleaned carefully to remove adhering fat body and muscle and rinsed extensively in Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). Samples of integument then were homogenized in TBS, using a motorized tissue grinder (Tissue Tearor, Biospec Products, Inc.). After centrifugation to remove insoluble material, samples of the supernatant were analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting, using antisera to prophenoloxidase isolated from hemolymph.8,14

Tyrosinase-type prophenoloxidase cDNA clones were obtained from an M. sexta hemocyte cDNA library by screening with antisera to prophenoloxidase.8,14 Laccase-type cDNA clones were obtained using polymerase chain reactions (PCR). Alignments of amino acid sequences of fungal laccases were used to identify conserved regions, which were used to design degenerate primers for PCR. These were used to amplify products from template DNA samples from M. sexta or Anopheles gambiae larval cDNA libraries. These products were cloned into plasmid vector pGem-T (Promega) and sequenced by the chain terminator method (Iowa State University DNA Sequencing Facility). Then they were labeled with 32P and used as probes for screening M. sexta and A. gambiae cDNA libraries in λZAP phage vectors (BioRad) using methods described previously.13,21 Sequences were used to search the non-redundant amino acid sequence database (National Center for Biotechnology Information) using the BLASTP program.22

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References


